



THE UNIVERSITY *of* EDINBURGH

This thesis has been submitted in fulfilment of the requirements for a postgraduate degree (e.g. PhD, MPhil, DClinPsychol) at the University of Edinburgh. Please note the following terms and conditions of use:

This work is protected by copyright and other intellectual property rights, which are retained by the thesis author, unless otherwise stated.

A copy can be downloaded for personal non-commercial research or study, without prior permission or charge.

This thesis cannot be reproduced or quoted extensively from without first obtaining permission in writing from the author.

The content must not be changed in any way or sold commercially in any format or medium without the formal permission of the author.

When referring to this work, full bibliographic details including the author, title, awarding institution and date of the thesis must be given.

**Phenotype and function of regulatory T cells in
Th1- and Th2-mediated inflammatory diseases.**

Dominika Joanna Nowakowska

A thesis submitted for the degree of Doctor of Philosophy

University of Edinburgh

2012

Declaration

I declare that this thesis has been submitted by myself, describes my own work and has not been submitted in any other application for a higher degree.

The cytokine bead array described in this thesis was performed by Dr Richard O'Connor.

Dominika Joanna Nowakowska

October 2012

Acknowledgements

Firstly, I would like to thank Steve for his support and guidance throughout my PhD. Thank you for your enthusiasm, encouragement and a positive attitude which helped a lot during difficult stages of quite a few of my experiments. Thank you to Rich O'C - you've been a great supervisor! Thank you for all your help, immense amount of patience, priceless advice and suggestions.

Thank you to the rest of Team A: Mel for not only helping me with experiments but also for making sure I didn't ask her more than one question a day - that was a steep learning curve but quickly made me independent! To everyone in my office: Rhoanne, Helen, Hayley, Stephanie and a new addition – Darryl. Thank you for all the fun, sugar and fat that you brought into my life. Beer/vodka & coke times in Bow bar and less posh places, weekly money moral dilemmas, cheesy TC music and lunches on the grass during Scottish summer times will be deeply missed and never forgotten! Thank you to the office guest, Ben, despite his numerous attempts to sabotage my experiments! I am sure psychic iTreg will rule science one day. Thank you to Rich Mellanby for helpful advice and always a cheerful attitude. Thank you to Cat who saved me so many times at the beginning of my PhD. To Teams B and C members, Siobhan and James, and Karen – thank you for helping me with crazy experiments that would otherwise see me working past midnight far too often! I will miss you guys! To Sarah – thank you so much that you were always there to help and give advice. Thank you to Fiona and Shonna for sorting my cells and putting up with my constant lack of punctuality, and to BRR staff.

Thank you to The Wellcome Trust who funded me through my PhD.

Thank you to everyone who made my time in Edinburgh so amazing and memorable, and to those who adopted me for long weekends/holidays. To my flatmates, Lisa and Annya – it's been great to share all the happy and sad moments with you. Last but not least, a big thank you goes to my parents and my brother for constant support, advice and keeping me sane in times of distress. You've been amazing and I wouldn't be at this stage if not you.

Abstract

Regulatory T cells (Treg) are critical to the maintenance of immune tolerance, partly by controlling the unwanted activation of effector T cells (Teff) and thereby enhancing the resolution of autoimmune and allergic inflammation. Recent data suggest that Treg can specialize to better control different types of inflammation by using transcriptional machinery which controls differentiation and function of Teff. This thesis addresses questions related to the efficacious use of Treg, notably their ability to adopt distinct phenotypic profiles under different inflammatory contexts and their need to recognize antigen in the inflamed organ.

Two differentially mediated mouse disease models were used in this project, namely Th1/Th17-mediated experimental autoimmune encephalomyelitis (EAE) as a model of multiple sclerosis and Th2-mediated allergic airways inflammation (AAI) as a model of asthma. A new model of rMOG-induced AAI was developed to specifically answer the questions on the importance of cell phenotype versus antigen-reactivity for the effective Treg-mediated suppression. It was demonstrated that Treg from the inflamed CNS in EAE had an upregulated expression of Th1 master regulator T-bet and Th1-associated chemokine receptor CXCR3, whereas Treg derived from the inflamed lung in AAI had an increased expression of Th2 master regulator GATA-3, lacked expression of T-bet and displayed decreased levels of CXCR3. This specialized and activated phenotype was restricted to tissue-derived Treg. The importance of appropriate Treg phenotype for effective suppression was suggested by the observed inability of CNS-derived Treg to inhibit AAI. A different Treg subset, TGF- β -induced Treg (iTreg), was shown to express high levels of T-bet and CXCR3, but not GATA-3 upon induction *in vitro*. iTreg effectively suppressed both Th1 and Th2 types of inflammation and the antigen-reactivity was key to this.

This thesis demonstrates that Treg are capable of acquiring a distinct phenotype corresponding with a CD4⁺ T cell response driving inflammatory disease and identifies antigen-reactivity as key to the efficacious suppression of inflammation. It also highlights substantial phenotypic differences between iTreg and naturally-occurring Treg which could be associated with different modes of suppression.

Posters and Presentations

Posters:

Dominika J Nowakowska, Richard A O'Connor, Melanie D Leech, Stephen M Anderton. Phenotypic characterization of Foxp3⁺ regulatory T cells in Th1- versus Th2-mediated inflammatory diseases. BSI/Edinburgh Immunology Group Annual Symposium, June 2011.

Presentations:

Invited Oral Presentation, World Immune Regulation Meeting-VI, Davos, Switzerland 2012. "Regulatory T cells specialize in allergic and autoimmune inflammation".

Table of Contents

Acknowledgements	iii
Abstract	iv
Posters and Presentations	v
Table of Contents	vi
List of Figures	xii
List of Tables	xv
List of Abbreviations	xvi

1 Introduction	1
1.1. Basic T cells biology	2
1.1.1. Generation in thymus	2
1.1.2. Positive selection	3
1.1.3. Negative selection	3
1.2. Development of immune responses	4
1.2.1. Innate immune system	4
1.2.2. Adaptive immune system	5
1.2.3. T cell activation	6
1.2.3.1. Signal 0: DC activation	6
1.2.3.2. Antigen processing and presentation	6
1.2.3.3. Signal 1: TCR engagement and signalling	7
1.2.3.4. Signal 2: Co-stimulation	8
1.2.3.5. Signal 3: Cytokines	9
1.3. Maintaining tolerance	10
1.3.1. Central tolerance	10
1.3.2. Peripheral tolerance	11
1.3.2.1. Death	11
1.3.2.2. Anergy / adaptation	11
1.3.2.3. Regulation	12
1.4. CD4 ⁺ T cell subsets	12
1.4.1. CD4 ⁺ effector T cells	13
1.4.1.1. Th1 cells	13
1.4.1.2. Th2 cells	14
1.4.1.3. Th17 cells	15
1.4.1.4. Novel CD4 ⁺ T cells subsets	16
1.4.2. CD4 ⁺ regulatory T cells	16
1.4.2.1. Natural Treg	17
1.4.2.1.1. nTreg generation	17
1.4.2.1.2. TCR signalling and reactivity	18
1.4.2.1.3. Cell surface markers	19
1.4.2.1.4. Foxp3 expression	19
1.4.2.2. Induced Foxp3 ⁺ Treg	21
1.4.2.2.1. Discovery of iTreg	21
1.4.2.2.2. Importance of TGF- β signalling	22
1.4.2.2.3. iTreg versus nTreg	22

1.4.2.3.	Tr1 and Th3 cells	23
1.4.2.4.	Other regulatory cell types	24
1.5.	CD4 ⁺ T cell plasticity	26
1.5.1.	Plasticity of cytokine production	26
1.5.2.	Flexibility in expression of master transcription factors.....	26
1.5.3.	Plasticity of Treg lineage	27
1.6.	Mechanisms of regulation by Treg	28
1.6.1.	Suppressive cytokines	29
1.6.2.	Cytolysis.....	30
1.6.3.	Metabolic disruption	30
1.6.4.	Modulation of APC.....	31
1.6.5.	Other mechanisms - adenosine.....	32
1.7.	Regulatory CD4 ⁺ T cells in autoimmune disease	34
1.7.1.	Multiple sclerosis	34
1.7.1.1.	Symptoms and clinical courses of MS	34
1.7.1.2.	Causes underlying MS	35
1.7.1.3.	Immunopathology	37
1.7.1.3.1.	CD4 ⁺ T cell-driven inflammation	37
1.7.1.3.2.	Impaired Treg regulation.....	40
1.7.1.4.	Current treatments.....	41
1.7.2.	EAE as a model of MS.....	42
1.7.2.1.	Immunopathology	43
1.7.2.2.	TCR transgenic models of EAE.....	46
1.7.2.3.	Regulation of EAE by Treg.....	47
1.7.2.4.	Therapeutic transfer of Treg.....	48
1.8.	Regulatory CD4 ⁺ T cells in allergy	49
1.8.1.	Asthma	49
1.8.1.1.	Symptoms and underlying causes of asthma	49
1.8.1.2.	Immunopathology of asthma.....	51
1.8.1.2.1.	CD4 ⁺ T cells orchestrate inflammatory reaction.....	51
1.8.1.2.2.	Breakdown in regulatory mechanisms	54
1.8.1.3.	Current treatments.....	54
1.8.2.	Allergic airways inflammation as a model of asthma.....	55
1.8.2.1.	Immunopathology	56
1.8.2.2.	Models of AAI	58
1.8.2.3.	Regulation of AAI by Treg	59
1.8.2.4.	Therapeutic transfer of Treg.....	61
1.9.	Treg in clinical trials	62
1.10.	Specialized subsets of Treg - do Treg adjust to the type of inflammation?	63
1.11.	Hypothesis and aims	65
2	Materials and Methods.....	66
2.1.	Mice.....	66
2.2.	Peptides and proteins.....	66
2.3.	Adjuvants	67
2.4.	General reagents.....	67
2.4.1.	Wash buffer.....	67
2.4.2.	RPMI-5% and RPMI-10%	67

2.4.3.	MACS buffer.....	67
2.4.4.	FACS buffer	67
2.4.5.	X-VIVO serum-free medium	67
2.5.	Cell isolation, purification and sorting.....	68
2.5.1.	Isolation of cells from spleen and lymph nodes.....	68
2.5.2.	Isolation of cells from CNS	68
2.5.3.	Isolation of cells from lung	68
2.5.4.	Purification of CD4 ⁺ T cells.....	69
2.5.5.	Purification of CD4 ⁺ CD62L ⁺ naïve cells by AutoMACS Pro Separator	69
2.5.6.	Sorting of CD4 ⁺ T cell subsets by FACS.....	69
2.5.6.1.	Sorting of nTreg from naïve Foxp3-GFP mice	70
2.5.6.2.	Sorting of naïve CD4 ⁺ CD62L ⁺ GFP ⁺ (Vβ11 ⁺ or Vα3.2 ⁺) cells. 70	
2.5.6.3.	Sorting of nTreg from the CNS/spleen of EAE mice and from naïve spleen for transfer	70
2.6.	<i>In vitro</i> cell culture and T cell polarisations	70
2.6.1.	Th1, Th2, Th17 and GM-CSF-polarising conditions.....	70
2.6.2.	Generation of iTreg	71
2.6.3.	Expansion of nTreg	71
2.6.4.	<i>Ex vivo</i> re-stimulation assays	72
2.6.5.	<i>Ex vivo</i> recall cytokine assays	72
2.6.6.	<i>In vitro</i> suppression assays	72
2.7.	<i>In vivo</i> manipulations	73
2.7.1.	Immunisations	73
2.7.2.	CD4 ⁺ T cell transfers.....	73
2.7.3.	<i>In vivo</i> CFSE-proliferation / suppression assays.....	73
2.7.4.	Induction and assessment of EAE.....	73
2.7.5.	Induction and assessment of rMOG-induced AAI.....	74
2.7.5.1.	Intraperitoneal sensitisation	74
2.7.5.2.	Th2 cell transfer	74
2.7.5.3.	Intratracheal instillation	74
2.7.5.4.	Bronchoalveolar lavage.....	75
2.7.5.5.	Histological scoring	75
2.8.	Depletion of Foxp3 ⁺ cells using DTx.....	76
2.9.	Enzyme Linked Immunosorbent Assays.....	76
2.9.1.	Cytokine quantification in cell supernatant.....	76
2.9.2.	Measurement of rMOG-specific IgE in serum.....	77
2.9.3.	Measurement of rMOG-specific IgG1 and IgG2a in serum	78
2.10.	Cytokine analysis using 13-plex Flowcytomix Multiplex	78
2.11.	Immunohistochemistry.....	78
2.12.	Flow cytometric analysis.....	79
2.12.1.	Antibodies for flow cytometric analysis	79
2.12.2.	Viability staining.....	79
2.12.3.	Surface staining.....	80
2.12.4.	Intracellular cytokine staining.....	80
2.12.5.	Intracellular staining for transcription factors.....	80
2.12.6.	Flow cytometric data analysis.....	81
2.13.	Statistics	81

3	Developing a model of rMOG-induced allergic airways inflammation	83
3.1.	Introduction	83
3.1.1.	Aims	84
3.1.2.	Experimental approach.....	84
3.2.	Results	85
3.2.1.	Sensitization with rMOG in alum and subsequent airway challenges do not induce AAI in C57BL/6 mice	85
3.2.2.	MOG-deficient mice develop eosinophilia but not lung inflammation upon sensitization and challenge with rMOG.....	86
3.2.3.	Optimisation of Th2 polarising protocol of MOG ₃₅₋₅₅ -reactive cells.	87
3.2.4.	Adoptive transfer of Th2 polarised 2D2 CD4 ⁺ cells followed by airway challenge induces AAI	88
3.2.5.	rMOG-induced AAI is characterized by increased frequency of Th2 2D2 cells within the lung and medLN	89
3.2.6.	Th2 2D2 cells have a stable phenotype <i>in vivo</i> following rMOG challenge	90
3.2.7.	Percentage and total number of Foxp3 ⁺ cells is elevated in the lungs of rMOG-challenged mice	91
3.2.8.	DTx administration protocol for depletion of Foxp3 ⁺ cells during AAI	92
3.2.9.	Host Foxp3 ⁺ CD4 ⁺ cells are important in controlling rMOG-induced AAI	93
3.2.10.	Depletion of host Foxp3 ⁺ cells is associated with increased frequency of Foxp3 ⁺ Th2 2D2 cells	94
3.3.	Discussion	117
3.3.1.	Why does rMOG sensitization do not induce lung inflammation?..	117
3.3.2.	How does rMOG-induced AAI mediated by Th2 2D2 cells compare to other models of AAI?.....	118
3.3.3.	A role for Foxp3 ⁺ cells in the control of lung inflammation in AAI	120
3.4.	Concluding remarks	123
4	Treg polarisation <i>in vivo</i> and <i>in vitro</i>.....	124
4.1.	Introduction	124
4.1.1.	Aims	125
4.1.2.	Approach	125
4.2.	Results	126
4.2.1.	Phenotypic analysis of Foxp3 ⁺ cells <i>in vivo</i>	126
4.2.1.1.	Upregulated expression of T-bet and CXCR3 in Foxp3 ⁺ cells from inflamed CNS in EAE	126
4.2.1.2.	Upregulated expression of GATA-3 and lack of T-bet in Foxp3 ⁺ cells from inflamed lung in AAI	128
4.2.2.	Foxp3 ⁺ T cell manipulation <i>in vitro</i>	129
4.2.2.1.	Lack of T-bet and low/intermediate levels of GATA-3 in Foxp3 ⁺ cells at a steady-state level.....	129
4.2.2.2.	nTreg upregulate T-bet and GATA-3 under Th1 and Th2 polarising conditions <i>in vitro</i> , respectively	129
4.2.2.3.	Expanding nTreg under polarising conditions	130

4.2.2.4.	TGF- β -induced Foxp3 ⁺ cells have a Th1-like phenotype	131
4.2.2.5.	Induction of iTreg under polarising conditions.....	132
4.2.2.6.	Re-stimulation of iTreg under polarising conditions	133
4.3.	Discussion	148
4.3.1.	Specialization of Treg in Th1- and Th2-mediated inflammation....	148
4.3.2.	Homing / activation status – tissue-derived versus peripheral Treg	151
4.3.3.	Cytokine control of Treg polarisation <i>in vitro</i>	152
4.3.4.	How do pro-inflammatory cytokines affect <i>in vitro</i> generated iTreg?	154
4.4.	Concluding synopsis	156
5	Functional consequences of Treg phenotype / polarisation	157
5.1.	Introduction	157
5.1.1.	Aims	158
5.1.2.	Approach	158
5.2.	Results	159
5.2.1.	Regulation by nTreg.....	159
5.2.1.1.	nTreg suppress naïve CD4 ⁺ T cells <i>in vitro</i>	159
5.2.1.2.	Suppression by nTreg expanded under polarising conditions..	159
5.2.1.3.	T-bet ⁺ CNS-derived Treg do not suppress rMOG-induced AAI	160
5.2.1.4.	Treg in Th1-mediated AAI do not acquire expression of T-bet	161
5.2.2.	Regulation by iTreg.....	162
5.2.2.1.	2D2 iTreg suppress proliferation of naïve 2D2 CD4 ⁺ T cells <i>in vitro</i> more effectively than polyclonal iTreg	162
5.2.2.2.	2D2 iTreg proliferate <i>in vivo</i> in response to antigen but partially lose Foxp3 expression	163
5.2.2.3.	2D2 iTreg limit the survival of naïve 2D2 cells and effector cytokine production <i>in vivo</i>	164
5.2.2.4.	2D2 iTreg increase Foxp3 expression within transferred 2D2 cells	165
5.2.2.5.	2D2 iTreg but not polyclonal iTreg suppress induction of EAE	165
5.2.2.6.	2D2 iTreg accumulate in dLN when transferred prior to EAE induction	167
5.2.2.7.	iTreg suppress proliferation and cytokine production by activated effector T cells <i>in vitro</i> in an antigen-dependent manner	168
5.2.2.8.	2D2 iTreg accumulate in CNS during EAE but are unable to suppress on-going inflammation	168
5.2.2.9.	2D2 iTreg migrate to the lung and medLN upon rMOG airway challenge	170
5.2.2.10.	iTreg suppress rMOG-induced AAI driven by pre-activated Th2 cells in antigen-dependent fashion	170
5.2.2.11.	Highest iTreg to Th2 cell ratio is found in the lung.....	172
5.2.2.12.	Induction of Foxp3 expression in Th2 2D2 cells by 2D2 iTreg	172
5.2.2.13.	2D2 iTreg do not suppress AAI driven by a different antigen.	173

5.2.2.14.	2D2 iTreg suppress Th2-associated cytokines in a bystander fashion in AAI.....	175
5.3.	Discussion	210
5.3.1.	Effect of Treg phenotype on suppression.....	210
5.3.2.	Effect of iTreg antigen reactivity on suppression	212
5.3.3.	How do iTreg suppress?.....	214
5.3.3.1.	Antigen-specific suppression of EAE induction.....	214
5.3.3.2.	Antigen-specific suppression of Th2-driven AAI.....	217
5.3.3.3.	'Infectious tolerance' as a mode of iTreg-mediated suppression?	218
5.3.3.4.	Bystander suppression in a model of AAI	219
5.3.4.	Stability of iTreg in inflamed environment.....	220
5.4.	Concluding synopsis	222
6	General discussion.....	224
6.1.	The importance of Treg phenotype and antigen reactivity in the control of inflammation	225
6.2.	Therapeutic application of iTreg in MS and asthma.....	227
6.3.	Future work	230
6.4.	Closing statement	231
7	References	232
8	Appendix.....	268

List of Figures

Figure 1.1 Signals required for CD4 ⁺ T cell activation.....	9
Figure 1.2 Key CD4 ⁺ T cell subsets.....	25
Figure 1.3 Mechanisms of Treg-mediated suppression.	33
Figure 1.4 Immune responses in MS.....	39
Figure 1.5 Immunopathogenesis of asthma.....	53
Figure 3.1 Sensitization and airway challenge with rMOG does not drive eosinophilia and lung inflammation in WT C57BL/6 mice.....	95
Figure 3.2 rMOG-specific IgE and IgG1 but not IgG2a are detected in serum from WT C57BL/6 mice sensitized and challenged with rMOG.	96
Figure 3.3 MOG-deficient mice develop lung eosinophilia but not lung inflammation.	97
Figure 3.4 rMOG specific IgG1 and IgE but not IgG2a are detected in serum from MOG-deficient mice sensitized with rMOG.....	98
Figure 3.5 Antigen challenge in rMOG sensitized MOG-deficient mice does not affect total CD4 ⁺ cells but significantly increases Foxp3 expression within the lung.	99
Figure 3.6 Optimisation of Th2 polarising conditions <i>in vitro</i>	100
Figure 3.7 Adoptive transfer of Th2 polarised 2D2 CD4 ⁺ cells followed by rMOG challenge induces eosinophils in BAL consistent with AAI.....	101
Figure 3.8 Th2-associated IL-13 and IL-5 are the predominant cytokines detectable in BAL fluid of rMOG-challenged mice.....	102
Figure 3.9 Th2 polarised 2D2 CD4 ⁺ T cells induce lung infiltration, goblet cell hyperplasia and lung eosinophilia upon airway challenge with rMOG.....	103
Figure 3.10 Increased total cell numbers in the lung and medLN, and an increased percentage of total CD4 ⁺ cells within the lung following transfer of Th2 2D2 cells and rMOG challenge.	104
Figure 3.11 Percentages and total cell numbers of Th2 2D2 cells increase upon rMOG challenge specifically within the lung and medLN.	105
Figure 3.12 Transferred Th2 2D2 cells retain the highest expression of GATA-3 in the rMOG-challenged lung.	106
Figure 3.13 Transferred Th2 2D2 cells from the challenged lung retain ability to produce Th2-associated IL-13.....	107
Figure 3.14 Percentages and absolute cell numbers of total Foxp3 ⁺ CD4 ⁺ cells increase upon rMOG challenge specifically within the lung.	108
Figure 3.15 Depletion of Foxp3 ⁺ cells following diphtheria toxin (DTx) treatment in Foxp3.LuciDTR4 mice during and throughout the induction of AAI.	109
Figure 3.16 Depletion of Foxp3 ⁺ cells prior to and during induction of AAI does not significantly affect the percentage and number of BAL eosinophils.....	110
Figure 3.17 Depletion of Foxp3 ⁺ cells resulted in the increased lung infiltration but did not affect percentages of goblet cells.	111
Figure 3.18 Depletion of Foxp3 ⁺ cells prior to and during AAI induction resulted in the increased percentage of total CD4 ⁺ cells within the lung.	112
Figure 3.19 Depletion of Foxp3 ⁺ cells prior to and during AAI induction did not affect percentages and total numbers of 2D2 Th2 cells within the lung and medLN.	113

Figure 3.20 Increased frequency of IL-13 ⁺ and GM-CSF ⁺ 2D2 Th2 cells from the lung of mice depleted of Foxp3 ⁺ cells.....	114
Figure 3.21 Administration of DTx prior to and during AAI induction did not affect the percentage and absolute numbers of total Foxp3 ⁺ CD4 ⁺ cells but decreased percentage of host Foxp3 ⁺ cells in the medLN.	115
Figure 3.22 Depletion of host Foxp3 ⁺ cells resulted in the increased frequency of Foxp3 ⁺ 2D2 Th2 cells in the medLN and spleen.	116
 Figure 4.1 Treg derived from the inflamed CNS in EAE have an upregulated expression of T-bet.....	134
Figure 4.2 Treg derived from the inflamed CNS in EAE express high levels of CXCR3 and CD73 and have an activated phenotype.	135
Figure 4.3 Treg derived from the inflamed lung in AAI do not express T-bet and have an upregulated expression of GATA-3.....	136
Figure 4.4 Treg derived from the inflamed lung in AAI express steady-state levels of CXCR3, but are CD73 ^{high} and have an activated phenotype.	137
Figure 4.5 Foxp3 ⁺ CD4 ⁺ cells at a steady state are T-bet ⁻ but express low/intermediate levels of GATA-3.....	138
Figure 4.6 Foxp3 ⁺ CD4 ⁺ cells stimulated <i>in vitro</i> upregulate T-bet and GATA-3 expression under Th1 and Th2 polarising conditions, respectively.....	139
Figure 4.7 Expansion of nTreg under polarising conditions.....	140
Figure 4.8 nTreg expanded under Th1 polarising conditions upregulate expression of T-bet and CXCR3 but maintain GATA-3.....	141
Figure 4.9 Generation and phenotype of iTreg.	142
Figure 4.10 Phenotype of <i>in vitro</i> generated iTreg.	143
Figure 4.11 IL-12 does not affect Foxp3 induction and T-bet expression in differentiating iTreg.	144
Figure 4.12 IL-4 inhibits Foxp3 induction in a dose-dependent manner and does not upregulate GATA-3 expression in iTreg at the time of induction.	145
Figure 4.13 Re-stimulation of iTreg under polarising conditions leads to rapid loss of Foxp3 expression which is prevented by exogenous TGF-β.	146
Figure 4.14 iTreg re-stimulated under Th1 or Th2 polarising conditions do not upregulate expression of T-bet or GATA-3, respectively.....	147
 Figure 5.1 Naïve nTreg suppress polyclonal proliferation of naïve CD4 ⁺ cells <i>in vitro</i>	176
Figure 5.2 nTreg expanded under Th1- and Th2-polarising conditions effectively suppress polyclonal proliferation of naïve CD4 ⁺ cells <i>in vitro</i>	177
Figure 5.3 nTreg expanded under neutral or polarising conditions do not differ in their ability to suppress pre-activated Th1 and Th2 cells.	178
Figure 5.4 CNS-derived Treg do not suppress BAL eosinophilia in rMOG-induced AAI.....	179
Figure 5.5 CNS-derived Treg do not suppress lung infiltration and goblet cell hyperplasia in rMOG-induced AAI.	180
Figure 5.6 Total cell numbers and percentages of Th2 2D2 cells in the lung and medLN are unaffected by CNS-derived Treg.	181
Figure 5.7 Adoptive transfer of Th1 2D2 cells followed by rMOG challenge induces neutrophils in BAL and lung infiltration but not goblet cell hyperplasia.	182

Figure 5.8 Transfer of Th1 2D2 cells induces an increase in the number of Foxp3 ⁺ cells within the lung however these do not express T-bet.	183
Figure 5.9 2D2 iTreg suppress proliferation of naïve 2D2 CD4 ⁺ cells <i>in vitro</i> more effectively than polyclonal iTreg.	184
Figure 5.10 2D2 iTreg proliferate <i>in vivo</i> in response to antigen and partially lose Foxp3 expression.	185
Figure 5.11 2D2 iTreg limit survival but not proliferation of naïve 2D2 CD4 ⁺ cells <i>in vivo</i> upon immunization.	186
Figure 5.12 2D2 iTreg reduce levels of effector cytokines produced in response to recall stimulation with antigen.	187
Figure 5.13 2D2 iTreg increase Foxp3 expression within transferred naïve 2D2 CD4 ⁺ cell population upon immunization.	188
Figure 5.14 2D2 iTreg but not polyclonal iTreg suppress induction of EAE.	189
Figure 5.15 2D2 iTreg transferred prior to immunization suppress production of pro-inflammatory cytokines within the CNS.	190
Figure 5.16 2D2 iTreg transferred prior to immunization reduce cytokine production within the dLN but not in the spleen.	191
Figure 5.17 2D2 iTreg accumulated within dLN and this corresponded with increased total cell numbers of CD4 ⁺ Foxp3 ⁺ cells.	192
Figure 5.18 2D2 iTreg suppress proliferation of pre-activated polarised CD4 ⁺ cells <i>in vitro</i> more effectively than polyclonal iTreg.	193
Figure 5.19 2D2 iTreg suppress cytokine production by pre-activated polarised CD4 ⁺ cells <i>in vitro</i> more effectively than polyclonal iTreg.	194
Figure 5.20 2D2 iTreg suppress GM-CSF production by Th1- and Th2-polarised cells but less effectively by GM-CSF-polarised cells.	195
Figure 5.21 2D2 iTreg do not protect from EAE when transferred after the onset of the disease.	196
Figure 5.22 2D2 iTreg accumulate in CNS during EAE but are unable to suppress on-going inflammation.	197
Figure 5.23 2D2 iTreg migrate to the medLN and the lung upon airway challenge with rMOG.	198
Figure 5.24 2D2 iTreg but not polyclonal iTreg reduce BAL eosinophilia driven by Th2 2D2 cells in rMOG-induced AAI.	199
Figure 5.25 2D2 iTreg reduce lung infiltration and goblet cell hyperplasia in rMOG-induced AAI.	200
Figure 5.26 2D2 iTreg reduce the numbers of Th2 2D2 cells within the lung and medLN.	201
Figure 5.27 2D2 iTreg suppress the production of Th2-associated cytokines and increase the production of IFN-γ.	202
Figure 5.28 The ratio of 2D2 iTreg to Th2 cells is the highest in the lung.	203
Figure 5.29 2D2 iTreg but not polyclonal iTreg increase the percentages of Foxp3 ⁺ cells within transferred Th2 2D2 cells.	204
Figure 5.30 2D2 iTreg but not polyclonal iTreg induce expression of Foxp3 in transferred Th2 2D2 cells.	205
Figure 5.31 2D2 iTreg reduce BAL eosinophilia driven by OVA-reactive Th2 CD4 ⁺ cells when iTreg cognate antigen is present.	206
Figure 5.32 2D2 iTreg do not suppress lung infiltration and goblet cell hyperplasia driven by Th2 OT-II cells.	207

Figure 5.33 The presence of the cognate antigen is critical for 2D2 iTreg migration into the lung and medLN.....	208
Figure 5.34 2D2 iTreg suppress the production of Th2 OT-II-derived IL-5 and IL-13.....	209

List of Tables

Table 2.1 Antibodies used for flow cytometry.....	82
Table 2.2 Capture and detection antibodies used for cytokine ELISA.	82

List of Abbreviations

AAI	Allergic airways inflammation
AHR	Airway hyperresponsiveness
Aire	Autoimmune regulator
APC	Antigen presenting cells
APS1	Autoimmune polyendocrinopathy syndrome type I
BAL	Bronchoalveolar lavage
BBB	Blood-brain-barrier
CCR-	Chemokine receptor-
CFA	Complete Freund's adjuvant
CFSE	Carboxyfluorescein succinimidyl ester
CNS	Central nervous system
CSF	Cerebrospinal fluid
cTEC	Cortical thymic epithelial cells
CTLA-4	Cytotoxic T-lymphocyte antigen-4
DC	Dendritic cells
DN	Double-negative
DP	Double-positive
DR5	Death receptor 5
DTR	Diphtheria toxin receptor
DTx	Diphtheria toxin
EAE	Experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
ELISA	Enzyme-linked immunosorbant assay
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
Foxp3	Forkhead box P3
GFP	Green-fluorescent protein
GITR	Glucocorticoid-induced TNF receptor-related protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GvHD	Graft versus host disease
GWAS	Genome-wide association studies
HDM	House dust mite
HEL	Hen egg lysozyme
HHV-6	Human herpes virus-6
IBD	Inflammatory bowel disease
ICS	Intracellular cytokine staining
IDO	Indoleamine 2,3-dioxygenase
IFN- γ	Interferon- γ
IL-	Interleukin-
ingLN	Inguinal lymph nodes
i.p.	Intraperitoneal
IPEX	Immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome
IRF-4	Interferon regulatory factor-4
i.t.	Intratracheal
ITAM	Immunoreceptor tyrosine-based activation motif

iTreg	Induced regulatory T cells
i.v.	Intravenous
LAG-3	Lymphocyte activation gene-3
LCMV	Lymphocytic choriomeningitis virus
LN	Lymph node
LPS	Lipopolysaccharide
LT	Lymphotoxin
MBP	Myelin basic protein
medLN	Mediastinal lymph nodes
MHC	Major histocompatibility complex
MOG	Myelin oligodendrocyte glycoprotein
MS	Multiple sclerosis
MTB	Mycobacterium tuberculosis
mTEC	Medullary thymic epithelial cells
NFAT	Nuclear factor of activated T cells
NK	Natural killer cells
nTreg	Natural regulatory T cells
OD	Optical density
OVA	Chicken ovalbumin
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PD-1	Programmed death-1
PFA	Paraformaldehyde
PLP	Proteolipid protein
PMA	Phorbol myristate acetate
PPMS	Primary progressive MS
PRR	Pattern recognition receptor
Ptx	Pertussis toxin
RAG	Recombination activating gene
ROR-	Retinoic acid-related orphan receptor-
RRMS	Relapsing-remitting MS
s.c.	Sub-cutaneous
SP	Single-positive
SPMS	Secondary progressive MS
STAT	Signal transducer and activator of transcription
V	Variable (region)
TCR	T cell receptor
Teff	Effector T cells
Tfh	T follicular helper cells
TGF- β	Transforming growth factor- β
Th	T helper
TLR	Toll-like receptor
TNF- α	Tumour necrosis factor- α
Tr1	T regulatory cell 1
TRAIL	TNF-related apoptosis-inducing ligand
Treg	Regulatory T cells
TSA	Tissue-specific antigens
WT	Wild-type

1 Introduction

Context

Immune regulation is essential to prevent excessive and unwanted immune responses directed at self- or innocuous antigens, and to promote a natural resolution of inflammation. Breakdown in immune tolerance and impaired regulation can lead to autoimmune and allergic diseases such as multiple sclerosis (MS) and asthma. Both MS and asthma are complex chronic inflammatory diseases believed to be coordinated by CD4⁺ T cells. MS is a demyelinating autoimmune disease of the central nervous system (CNS) mediated by myelin-reactive T helper (Th)1 and Th17 cells (Sospedra and Martin, 2005). Asthma is a lung inflammatory disease mediated by Th2-driven IgE responses to otherwise harmless allergens which results in airway remodelling (Wills-Karp, 1999). Pathogenesis in both diseases is multifactorial, with complex interactions of genetic and environmental links.

Foxp3⁺ regulatory T cells (Treg) have been demonstrated to effectively suppress CD4⁺ T cell responses via a variety of mechanisms (Shevach, 2009). Their importance is highlighted by the fact that a natural mutation in the *foxp3* gene leads to autoimmune lymphoproliferative disorders in both mice and humans (Bennett et al., 2001, Brunkow et al., 2001). Evidence of impaired Treg function in MS and asthma patients has led to the investigation of this cell population in the context of animal models of human disease: experimental autoimmune encephalomyelitis (EAE) which is a model of MS and allergic airways inflammation (AAI) which is a model of allergic asthma. In both models pathogenic CD4⁺ T cell responses have been demonstrated to be regulated by Treg (Kohm et al., 2002, Kearley et al., 2005) and protective effects of Treg on disease severity suggested their potential use in immunotherapy.

This thesis addresses questions related to the efficacious use of Treg, notably their ability to adopt distinct phenotypic profiles under different inflammatory contexts and their need to recognize antigen in the inflamed organ.

1.1. Basic T cells biology

Lymphocytes develop in the bone marrow from pluripotent hematopoietic stem cells that by division produce two types of stem cells: common lymphoid progenitor and common myeloid progenitor. Common lymphoid progenitor gives rise to T cells, B cells and natural killer (NK) cells which further differentiate at distinct sites. Progenitor cells that generate T cells migrate from the bone marrow to thymus which provides a specialised microenvironment for their maturation (Petrie and Zuniga-Pflucker, 2007).

1.1.1. Generation in thymus

There are two separate T cell lineages that are generated in thymus and these are defined by their differential expression of an $\alpha\beta$ T cell receptor (TCR) or a $\gamma\delta$ TCR (Ciofani and Zuniga-Pflucker, 2010). During maturation, T cells pass through several stages of development, the earliest being the double-negative (DN, $CD4^-CD8^-$) stage subdivided into DN1, DN2, DN3a and DN3b stages. During the progression from the DN1 to DN3a stage, β -, γ - and δ -chain rearrangements occur and by the DN3b stage a decision is made whether a thymocyte becomes an $\alpha\beta$ T cell or a $\gamma\delta$ T cell (Burtrum et al., 1996, Livak et al., 1999). At all stages cells receive rescue or apoptosis signals which drive further maturation or terminate cell fate. Thymocytes that successfully rearranged $\gamma\delta$ chains become mature $\gamma\delta$ T cells, whereas thymocytes that successfully rearranged VDJ segments of a β -chain become double-positive (DP, $CD4^+CD8^+$) and start rearrangement of an α -chain. Once a functional α -chain is produced and paired with β -chain, DP thymocytes undergo selection for their ability to recognize peptide-MHC complexes that yields mature $CD4^+$ or $CD8^+$ single-positive (SP) T cells.

1.1.2. Positive selection

Once an $\alpha\beta$ TCR is successfully rearranged, the processes of positive and negative selection are used to shape the mature TCR repertoire. DP thymocytes interact with thymic cortical epithelial cells (cTEC) that express self-peptides in a major histocompatibility complex (MHC)-restricted manner. The specificity of TCR determines whether a DP thymocyte will engage with the MHC class I-presented peptide and hence become a $CD8^+$ T cell, or with the MHC class II- presented peptide to become a $CD4^+$ T cell (Kisielow et al., 1988, Scott et al., 1989). Only thymocytes that recognize self-peptides in a relevant MHC context receive survival signals and proceed to the next stage of selection (Jameson et al., 1995).

1.1.3. Negative selection

Random rearrangements of α - and β -chains provide not only a great diversity in the TCR repertoire but also a danger of generating potentially harmful autoreactive T cells. In a process of negative selection, $CD4^+$ and $CD8^+$ SP T cells move from the cortex into the medulla and interact with medullary TEC (mTEC) and bone marrow-derived antigen presenting cells (APC) such dendritic cells and macrophages (Zoller, 1991). If the TCR reacts strongly with self-peptide-MHC complex on an mTEC or APC, a T cell is deleted (Zal et al., 1994, Douek et al., 1996). This ensures that all T cells that react strongly to self-peptides are removed from a general T cell cohort. Once SP T cells have passed through both selection processes and are fully matured, they enter the blood system and are carried to the peripheral (secondary) lymphoid tissues.

Both positive and negative selection are critical in generating T cells that respond in an MHC-restricted manner and are depleted of strongly autoreactive cells before entering the periphery. This is the basis of central tolerance which will be discussed in more detail later on in this chapter.

1.2. Development of immune responses

Pathogens can enter the body by many different routes and to provide protection from infections, antigens are constantly being sampled by antigen presenting cells and carried to the secondary lymphoid tissues. Mature T cells exit the thymus and recirculate continuously between the peripheral lymphoid organs and blood until they encounter their cognate antigen. This interaction is facilitated in the secondary lymphoid organs which integrate both innate and adaptive immune system components and enable an adaptive immune response to be initiated.

1.2.1. Innate immune system

The innate immune system forms not only a second line of defence against pathogens (the first being epithelium) but also plays a critical role in initiating the adaptive immune response. Innate immune system includes mast cells, neutrophils, eosinophils, basophils, phagocytic cells such as macrophages and complement proteins. The cellular components are all derived from a common myeloid progenitor stem cell and mature in the bone marrow. Mast cells, basophils and eosinophils are large secretory cells that upon activation release granules containing toxic and pro-inflammatory molecules such as histamine, cytokines and cationic proteins (O'Donnell et al., 1983, Welle, 1997). These cells are very important in defence against extracellular parasites but can also orchestrate unwanted allergic responses (Stone et al., 2010).

Macrophages and neutrophils are professional phagocytic cells able to phagocytose and kill extracellular pathogens. Macrophages act in tissue as sentinel cells sampling the environment for pathogens. Once they are activated by danger signals, they increase their rate of phagocytosis and secrete cytokines which attract neutrophils from blood (Silva, 2010). Neutrophils are short-lived and can efficiently kill pathogens by phagocytosis and production of antimicrobial products (Nathan, 2006). The process of phagocytosis is greatly facilitated by complement proteins and antibodies which opsonize the pathogen and bind to phagocytes via Fc/complement-receptors on the cell surface (Carroll, 2004).

Another important function of macrophages but also of dendritic cells (DC) is to bring the innate and adaptive immune system together by acting as APC. Both macrophages and DCs have the ability to process protein antigens and present the resulting peptides in an MHC-restricted manner and provide co-stimulation to activate naïve T cells. Moreover, they play an important role in decision making on the type of immune response that is elicited (Kaiko et al., 2008).

The rapid response of the innate immune system provides many advantages to protection from common pathogens. However, more tuned and specific immune responses are needed along with immunological memory to combat the diverse range of continuously evolving pathogens, and this is where the adaptive immune system comes into play.

1.2.2. Adaptive immune system

The cells of the adaptive immune system, T cells and B cells, are generated to specifically recognize a wide range of pathogens with a potential $\alpha\beta$ TCR diversity of 10^{15} (Davis and Bjorkman, 1988). Appropriate and efficient activation of T cells and B cells requires highly organized secondary lymphoid structures. Mature antigen-inexperienced T cells (naïve T cells) enter secondary lymphoid organs such as lymph nodes via the high endothelial venules. Within a lymph node T cells accumulate in T cell zones, situated in the cortex, where they encounter antigens presented by APC. The transient binding of naïve T cells to APC enables them to sample peptide-MHC complexes on the surface of APC (Bousso, 2008, Ingulli et al., 2002). The majority of T cells do not encounter their cognate antigen, move into the medulla and leave the lymph node via efferent lymphatic vessel before returning to the circulation. If, however, a naïve T cell recognizes its antigen, a signalling cascade is activated which eventually leads to T cell activation and expansion (Stoll et al., 2002, Miller et al., 2004).

1.2.3. T cell activation

Bretscher and Cohn originally suggested a 2-signal model for lymphocyte activation: the first being a recognition of a cognate antigen and the second a co-stimulation (Bretscher and Cohn, 1970). This explained how a lymphocyte is activated in an antigen-specific way and can discriminate between self and non-self. Since then detailed studies into CD4⁺ T cell activation led to the redefinition of this model to include 3 signals: 1) activation through the TCR that recognizes the peptide-MHC complex on the surface of the APC, 2) co-stimulation through the accessory molecules on the surface of the T cell and APC and 3) cytokine signalling. However for this to occur, APC has to become activated and this is defined as signal 0.

1.2.3.1. Signal 0: DC activation

Although both macrophages and DC can present antigen to T cells, DC are most efficient at presenting exogenous antigens and activating naïve T cells (Steinman, 1991, de Jong et al., 2006). Immature DC frequently reside within peripheral tissues where they continuously take up foreign material. In the presence of danger signals, they become activated and migrate to the draining lymph nodes where they initiate T cell activation (Guermonprez et al., 2002). Danger signals include pathogen-associated molecular patterns (PAMPs) such as bacterial lipopolysaccharides (LPS) or viral-derived dsRNA (Bianchi, 2007) but also components released from necrotic cells, lysates such as uric acid (Shi et al., 2003). These are sensed by the DC via a wide variety of cell surface receptors, for example, pattern recognition receptors (PRR) including Toll-like receptors (Takeuchi and Akira, 2010).

1.2.3.2. Antigen processing and presentation

There are two main antigen processing pathways: 1) MHC class I-processing pathway which delivers peptides originating from the cytosol of the cell, occurs in all nucleated cells and the final product is recognized by CD8⁺ T cells, and 2) MHC class II-processing pathway which delivers peptides originating from endocytosed material, occurs only in APC and the peptide-MHC complex is recognized by CD4⁺

T cells (Vyas et al., 2008). MHC class II-processing pathway will be discussed briefly as it is critical to the activation of naïve CD4⁺ T cells.

Antigen taken up by the APC enters endosomes which become acidified as they progress towards the endoplasmic reticulum of the cell. They fuse with lysosomes containing proteases which cleave the protein into peptides. The vesicle then fuses with another vesicle pre-loaded with MHC class II molecule. The binding groove of the MHC class II molecule is occupied with an invariant chain that prevents inappropriate peptide-binding. It is cleaved by proteases upon fusion leaving only a small fragment bound to the MHC groove (CLIP). The removal of CLIP and the peptide loading is catalysed by a specialised MHC class II-like molecule called HLA-DO (H-20 in mice). The peptide-MHC II complex is next delivered to the cell surface.

1.2.3.3. Signal 1: TCR engagement and signalling

TCR is a heterodimer of an α -chain and β -chain linked by a disulphide bond. Each consists of an extracellular domain containing variable (V) regions responsible for antigen binding and a short intracellular domain. $\alpha\beta$ heterodimers are associated with CD3 which is a complex of four signalling chains (two ϵ , γ and δ) and a homodimer of ξ signalling chains. Each CD3 chain has a cytoplasmic tail containing one immunoreceptor tyrosine-based activation motif (ITAM) and each ξ chain has 3 ITAMS (Kuhns et al., 2006). The TCR complex is thought to consist of two $\alpha\beta$ heterodimers and 6 accessory chains and is associated with a CD4 or a CD8 co-receptor which binds to the invariant site of the MHC class II or MHC class I, respectively, and is required for stable peptide-MHC-TCR binding (Yin et al., 2012).

TCR engagement with the peptide-MHC complex leads to the clustering of the TCR complex and the co-receptors on the cell surface forming an immunological synapse (Grakoui et al., 1999). Activated Src-family protein kinases, Lck and Fyn, phosphorylate ITAMs on the CD3 and ξ chains allowing tyrosine kinase ZAP-70 to bind to the phosphorylated ITAMs on ξ chains. Activation of ZAP-70 initiates a

cascade of signalling events eventually leading to the activation of transcription factors NF κ B, NFAT and AP-1 which induce cell proliferation and differentiation (Nakayama and Yamashita, 2010).

1.2.3.4. Signal 2: Co-stimulation

For naïve T cells to be activated, in addition to TCR engagement with the peptide-MHC complex, there is a requirement for the co-stimulatory signals. TCR ligation in the absence of co-stimulation leads to the state of unresponsiveness, known as anergy (Schwartz, 2003).

One of the best characterised co-stimulatory molecules is CD28 which is constitutively expressed on both naïve and activated T cells (Gross et al., 1992). CD28 is a receptor that binds to CD80 (B7.1) or CD86 (B7.2) expressed on the surface of APC and is upregulated upon APC activation (signal 0) (Freeman et al., 1991). In naïve T cells signalling through CD28 reduces the threshold for T cell activation (Viola and Lanzavecchia, 1996) and promotes T cell survival (Sperling et al., 1996), whereas in previously activated T cells, it promotes IL-2 and effector cytokine production, cell proliferation and survival (Thompson et al., 1989, Boise et al., 1995). Other co-stimulatory molecules include CD40/CD40L, OX40/OX40L and ICOS/ICOSL (Sharpe and Freeman, 2002).

A number of co-inhibitory signals also exist and are critical for keeping T cells in check. These include molecules such as cytotoxic T-lymphocyte antigen-4 (CTLA-4) and programmed death-1 (PD-1) which are upregulated upon T cell activation. CTLA-4 competes for the same ligands as CD28 (Linsley et al., 1992) with higher affinity and its binding significantly impairs IL-2 production and T cell proliferation (Krummel and Allison, 1995). PD-1 has two differentially expressed ligands (PDL-1 and PDL-2) and was shown to be important for negative regulation of cell proliferation and cytokine production (Freeman et al., 2000).

1.2.3.5. Signal 3: Cytokines

The third signal, which is not required for T cell activation but is critical during the activation process and affects T cell differentiation, comes from cytokines produced by the APC. Upon encountering an antigen in the presence of danger signals such as PAMPs, APC are activated and can produce a wide range of cytokines which dictate what type of effector T cell (Teff) response will be elicited (Kapsenberg, 2003). Cytokines bind to their cell surface receptors on T cells and induce intracellular signalling which results in the activation of specific signal transducer and activator of transcription (STAT) proteins (Adamson et al., 2009). Activation of different STATs promotes expression of distinct genes directing an activated T cell (Th0) towards different differentiation pathways such as Th1, Th2 or Th17 (O'Shea and Paul, 2010) which are discussed later in section 1.4.

In a summary, signals 0, 1 and 2 ensure that only T cells recognizing specific pathogen-derived antigens are activated (and negatively regulated when needed) and signal 3 tunes the response to the type of invading pathogen (Figure 1.1). However, T cells recognizing self- or innocuous antigens can still be activated in the periphery and therefore various mechanisms of maintaining tolerance have evolved.

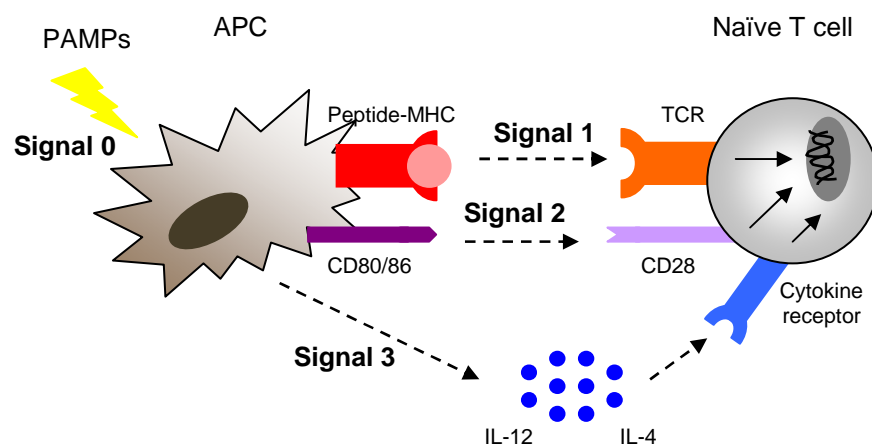


Figure 1.1 Signals required for CD4⁺ T cell activation.

CD4⁺ T cells require 4 signals for activation and differentiation. Signal 0: activation of APC by danger signals such as pathogen-associated molecular patterns (PAMPs). Signal 1: TCR engagement by peptide-MHC complex. Signal 2: co-stimulation. Signal 3: cytokine signalling which is not required for T cell activation but is critical for T cell differentiation.

1.3. Maintaining tolerance

Tolerance is required to prevent autoimmune diseases and chronic inflammation, and it can be maintained at 2 main levels: 1) central (in thymus) during maturation of naïve lymphocytes and 2) peripheral (in secondary lymphoid organs or tissues) when T cells that escaped central deletion are activated in the presence of self-reactive or innocuous antigens.

1.3.1. Central tolerance

Negative selection that occurs in thymus during maturation of lymphocytes is called central tolerance. As described before, T cells that bind to self-peptide-MHC complexes too strongly undergo apoptosis. The main and unique cell type that instructs T cells in this process is called medullary thymic epithelial cell (mTEC) which in addition to presenting ubiquitously expressed self-antigens, can also present tissue-specific antigens (TSA) (Derbinski et al., 2001). This promiscuous (ectopic) gene expression was discovered to be driven by Autoimmune regulator (*Aire*) (Liston et al., 2003), the gene that is responsible for a clinical disorder called autoimmune polyendocrinopathy syndrome type I (APS1) (Nagamine et al., 1997). Ectopic gene expression allows for T cells highly reactive to self-antigens not normally expressed in thymus to be deleted before they enter peripheral pool of naïve T cells. However, this mechanism is not fail-proof and not all peptides are presented efficiently by mTEC and hence self-reactive T cells can escape thymic deletion (Anderton and Wraith, 2002). This has been demonstrated to be true for encephalitogenic Ac1-9 peptide of myelin basic protein (MBP) which was shown to be expressed in thymus, however, this did not lead to deletion of T cells that can recognize it (Liu et al., 1995).

The existence of self-reactive T cells in the periphery is one of the reasons for the requirement of a second level of tolerance induction called peripheral tolerance.

1.3.2. Peripheral tolerance

Harmful immune reactions can not only be mediated by self-reactive T cells that escaped central tolerance, but can also occur due to the degeneracy of TCR recognition (Reiser et al., 2003). The ability of the TCR to recognize more than one antigen provides more robust protection against a wide variety of pathogens, however, it also means that a T cell can cross-react with a self-antigen. This is the basis of the molecular mimicry theory which suggests that self-antigens can contain similar epitopes to those found in pathogen-derived antigens and self-reactive T cells can therefore be initially activated during infection, ultimately leading to autoimmune response (Oldstone, 1987). Inducing tolerance is also very important for T cells recognizing innocuous antigens. Cells at the mucosal surfaces such as in the lung and the gut are in constant contact with harmless antigens and peripheral tolerance prevents a state of chronic inflammation. There are three main mechanisms of peripheral tolerance and these include death, adaptation and regulation (Ryan et al., 2007).

1.3.2.1. Death

High affinity TCR-bearing T cells that escape negative selection in the thymus and later become activated by a strong antigenic stimulus in the periphery can undergo apoptosis (Anderton et al., 2001, Wells et al., 1999). T cell death can also occur during activation by immature DC in the absence of co-stimulatory signals (Vella et al., 1995) through failure of upregulating anti-apoptotic signalling via Bcl-2, Bcl-xL and NF κ b (Bansal-Pakala et al., 2001) or downregulating pro-apoptotic signalling via Fas, FasL and Bim (Mueller, 2010).

1.3.2.2. Anergy / adaptation

Originally the term anergy was used to describe a state of unresponsiveness in T cells that had been stimulated *in vitro* with insufficient co-stimulation (Jenkins et al., 1987). This was found to result in reversible inhibition of IL-2 secretion and cell proliferation but did not affect T cell ability to produce effector cytokines (Mueller et al., 1989, Schwartz, 2003). How this observation reflects *in vivo* situation remains

unclear. A different form of anergy is observed *in vivo* and is called adaptive tolerance. It results in a complete inhibition of both cell proliferation and effector cytokine production and requires persistent presence of the antigen (Rocha et al., 1993, Singh and Schwartz, 2003).

1.3.2.3. Regulation

The third mechanism of peripheral tolerance is mediated by regulatory T cells (Treg). There are several subsets of Treg and these include naturally-occurring Foxp3⁺ Treg (nTreg) originating from the thymus, Foxp3⁺ peripherally-induced Treg (iTreg) and also less well-characterized subsets such as Tr1 and Th3 which are Foxp3 negative. Treg subsets and their mechanisms of suppression will be discussed later on in this chapter. The importance of Foxp3⁺ Treg is emphasized by the naturally occurring mutation within the *foxp3* gene that results in the development of severe multi-organ autoimmune disease in both human and mouse (Brunkow et al., 2001, Le Bras and Geha, 2006).

The so-called ‘three pillars’ of immune tolerance provide a safety turn-off mechanism at different stages of T cell responses and very rarely breakdown. The breakdown of immune tolerance can result in autoimmunity and/or chronic inflammation.

1.4. CD4⁺ T cell subsets

Activated T cells can follow multiple differentiation pathways and the choice of the pathway is dictated by the APC-derived cytokines that are produced during T cell stimulation. In 1986 Mosmann and Coffman first described two types of antigen-specific murine T cell clones that were defined by the cytokines they secreted and these two subsets were called Th1 and Th2 cells (Mosmann et al., 1986). Since then more CD4⁺ T cell subsets have been identified and these broadly include effector T cells and regulatory T cells. Each of the CD4⁺ T cell subsets is described in greater detail below and is illustrated in Figure 1.2 at the end of this section.

1.4.1. CD4⁺ effector T cells

Different forms of infectious pathology require specialised T cell responses involving expression of appropriate homing molecules and cytokines to be able to effectively steer the immune response to fight off the infection. However, due to their potent effector mechanisms Teff also became prominent for their contribution to inflammatory diseases such as rheumatoid arthritis and asthma (Brennan et al., 2002, Medoff et al., 2008). The main Teff subsets include Th1, Th2 and the more recently discovered Th17 cells.

1.4.1.1. Th1 cells

Th1 cells were originally defined by their ability to produce interferon- γ (IFN- γ) (Mosmann et al., 1986) with tumour necrosis factor- α (TNF- α) and lymphotoxin (LT) discovered later as the other two signature cytokines (Wan, 2010). One of the most important functions of Th1 cells is stimulation of innate responses to intracellular bacteria. Th1 cells were also shown to have a pathogenic potential and contribute to autoimmune diseases such as multiple sclerosis (Lassmann and Ransohoff, 2004).

In vitro studies have shown that differentiation of Th1 cells is induced by IL-12 (Hsieh et al., 1993, Seder et al., 1993) and enhanced by IL-18 (Yoshimoto et al., 1998). The requirement for IFN- γ is still unclear (Wang et al., 1994, Wenner et al., 1996). Binding of IL-12 to its receptor on the surface of a T cell initiates signalling that activates STAT4, a transcription factor that is required for differentiation of Th1 cells (Kaplan et al., 1996). One of its most important target genes is *tbx21* which encodes T-bet, a master regulator of Th1 lineage (Szabo et al., 2000). T-bet drives expression of IFN- γ and other Th1-associated cytokines, and also of chemokine receptors such as CXCR3 and CCR5. IFN- γ secreted by Th1 cells can act in an autocrine fashion by enhancing T-bet expression via STAT1 (Afkarian et al., 2002) and in a paracrine fashion by acting on Th2 cells and limiting their proliferation (Oriss et al., 1997).

In addition to secreting IFN- γ , Th1 cells also express a co-stimulatory ligand CD40L which can bind to the surface receptor of the macrophage. IFN- γ and CD40 ligation activate macrophages making them more effective at destroying intracellular pathogens such as *Mycobacterium* or *Leishmania* via production of TNF- α and nitric oxide (Salgame, 2005). The macrophage also upregulates the expression of MHC class II molecules and CD40 enabling it to efficiently re-activate rested T cells at the site of inflammation. Th1 cells can also express FasL promoting apoptosis of target cells and produce interleukin (IL)-2, IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF) important for maturation and activity of DC (Miller et al., 2002). The role of Th1 cells in driving pathogenic responses in autoimmune disease will be discussed later in this chapter.

1.4.1.2. Th2 cells

Th2 cells were identified at the same time as Th1 cells in 1986 (Mosmann et al., 1986) and their signature cytokines include IL-4, IL-5, IL-13 and IL-10. Th2 cells are specialized to activate B cells and hence induce antibody responses required for clearance of extracellular pathogens. Th2 cells are also very important at maintaining mucosal immunity in the lung and their aberrant responses can drive chronic inflammation which is observed in asthma (Robinson et al., 1992).

Differentiation of Th2 cells requires the presence of IL-4 which via STAT6 signalling induces high expression of GATA-3 binding protein (GATA-3), a key transcription factor reported to be necessary and sufficient for commitment to Th2 lineage (Zheng and Flavell, 1997). GATA-3 induces and stabilizes expression of IL-4, IL-5 and IL-13 (Zhang et al., 1997) and is thought to affect expression of Th2-associated chemokine receptors such as CCR3 and CCR4. GATA-3 expression can also be induced in IL-4- and STAT6-independent way via STAT5 signalling (Zhu et al., 2003). Moreover, low expression of GATA-3 is important during T cell development (Ting et al., 1996) and is maintained in naïve T cells (Zhang et al., 1997). Similar to IFN- γ in Th1 cells, IL-4 can act in an autocrine and paracrine

fashion by promoting Th2 responses and inhibiting Th1 responses (Wurtz et al., 2004).

One of the most important Th2 cell effector function is promoting B cell activation and antibody production to combat extracellular parasites. In addition to producing cytokines, Th2 cells express CD40L on their surface which binds to CD40 on the surface of a B cell and together with cytokines induces B cell proliferation and antibody class switching (Parker, 1993). Th2 cells can also produce eotaxin recruiting eosinophils from the bone marrow which upon activation degranulate releasing toxic substances able to kill parasites (Klion and Nutman, 2004). The powerful mechanisms of dealing with extracellular pathogens can sometimes become dysregulated and turn against the host leading to chronic inflammation which is seen, for example, in asthma. The role of Th2 cells in allergic inflammatory diseases will be discussed later in this chapter.

1.4.1.3. Th17 cells

The original Th1/Th2 paradigm, whereby these two cell populations have a potential to cancel each other out as a means of immune regulation, was broken by the discovery of a new CD4⁺ T cell subset called Th17 (Langrish et al., 2005, Bettelli et al., 2007). Signature cytokines of Th17 cells include IL-17A, IL-17F, IL-21 and IL-22 and appear to be important for the protection against mucosal pathogens such as fungi (Kolls, 2010).

The differentiation of Th17 cells requires IL-6 and TGF- β signalling via Smad2 and STAT3 (Mangan et al., 2006, Malhotra et al., 2010, Zhou et al., 2007). This induces an expression of a master regulator of Th17 differentiation called retinoic acid-related orphan receptor- γ T (ROR- γ T) (Ivanov et al., 2006). Another related nuclear receptor, ROR- α , was also shown to be required for Th17 differentiation (Yang et al., 2008b). IL-23 initially thought to be required for Th17 differentiation, was shown to stabilise the Th17 phenotype instead (Stritesky et al., 2008). The differentiation of Th17 cells is inhibited by Th1-associated IFN- γ and Th2-associated IL-4 (Weaver et

al., 2007). Th17 cells also seem to exhibit high level of plasticity in terms of cytokine production (Lee et al., 2009) and conversion from Foxp3⁺ cells (Xu et al., 2007).

Since their discovery, Th17 cells have been implicated in many autoimmune diseases (Lubberts, 2008, Durelli et al., 2009) and previously unclear evidence like exacerbation of 'Th1-mediated' autoimmune models with anti-IFN- γ therapy (Billiau et al., 1988) shed a new light on the pathogenesis in many diseases.

1.4.1.4. Novel CD4⁺ T cells subsets

Among the newly defined Teff subsets are T follicular helper cells (Tfh) which are antigen-experienced CD4⁺ T cells residing in B cell follicles in the secondary lymphoid organs. These cells are characterized by the expression of CXCR5 (Schaerli et al., 2000) and the transcription factor Bcl6 (Nurieva et al., 2009) and aid the formation of B cell germinal centres (Crotty, 2011).

The two other new subsets of Th cells include IL-9-secreting Th9 cells (Dardalhon et al., 2008) and IL-22-secreting Th22 cells (Trifari et al., 2009, Eyerich et al., 2009), however their role is still unclear and no master transcription factors driving their lineage have been yet defined.

1.4.2. CD4⁺ regulatory T cells

In addition to effector T cells eliciting immune responses during infections and inflammatory diseases there are several subsets of CD4⁺ T cells that are immunosuppressive and aim to dampen these responses. The existence of 'suppressive' T cells was originally suggested in early 1970s by Gershon and Kondo who reported that antigen tolerance required the presence of thymus-derived lymphocytes (Gershon and Kondo, 1970, Gershon and Kondo, 1971). Since the discovery of a master transcription factor that drives regulatory T cell lineage, Forkhead box P3 (Foxp3) in 2003 by the Sakaguchi group, the understanding of these cells has greatly progressed (Hori et al., 2003). It is now known that there are

two main subsets of Treg and these include naturally-occurring Treg (nTreg) and induced Treg (iTreg) that can be generated either in the periphery or *in vitro*.

1.4.2.1. Natural Treg

The first classification of regulatory T cells as a specific T cell subset was reported by Sakaguchi et al. who showed that mice depleted of T cells expressing the high affinity IL-2 receptor α -chain (IL-2R α , also called CD25) develop severe multi-organ autoimmunity (Sakaguchi et al., 1985). Reciprocally, the reconstitution with CD25⁺ T cells prevented disease development by limiting the responses to both self- but also to non-self antigens (Sakaguchi et al., 1995). Neonatal thymectomy and cell transfer experiments showed that CD25⁺ T cells, contained mostly within CD4⁺ cell population, are generated in thymus from day three onwards in mice and are responsible for maintaining peripheral tolerance (Asano et al., 1996).

1.4.2.1.1. nTreg generation

nTreg constitute approximately 5-10% of peripheral CD4⁺ T cells and are generated as a distinct lineage during thymic development (Itoh et al., 1999). Processes of positive and negative selection in thymus are based on the strength of antigen-self-reactivity. As mentioned already, positive selection allows for survival of T cells weakly recognizing peptide-MHC complexes, whereas negative selection deletes the cells that bind to peptide-MHC complexes with too high affinity. Due to controversy over how self-reactive the TCR repertoire of nTreg is (Pacholczyk and Kern, 2008), the thymic selection of nTreg is still not thoroughly understood. An instructive model suggests that nTreg are selected on the basis of high affinity TCR for the self-peptide and this is an alternative fate to deletion during negative selection (Jordan et al., 2001, Yu et al., 2008). A different, 2-step model, proposes that nTreg selection is random and involves an initial upregulation of CD25 on single-positive thymocytes which is followed by the expression of Foxp3 (van Santen et al., 2004, Lio and Hsieh, 2008). Signalling through TGF- β and CD28 is suggested to be critical as well (Liu et al., 2008, Tai et al., 2005). It was reported that, once nTreg leave the thymus,

their full development and/or survival is dependent on the presence of peripheral self-antigen (Modigliani et al., 1996, Seddon and Mason, 2000).

1.4.2.1.2. TCR signalling and reactivity

TCR signalling is critical for development and function of nTreg. TCR transgenic mice that lack recombination activating gene (RAG) responsible for VDJ-rearrangements, have no nTreg. Moreover, the number of nTreg in TCR transgenic mice is lower than in wild type mice suggesting that certain $\alpha\beta$ TCR are required for optimal nTreg generation (Olivares-Villagomez et al., 2000). Development of *in vitro* Treg suppression assay by the Shevach group greatly facilitated understanding of the importance of Treg antigen reactivity and the mechanisms of suppression (Thornton and Shevach, 1998, Thornton and Shevach, 2000). The suppression assay consists of an unchanged number of CD4⁺CD25⁻ conventional T cells (Teff) and APC incubated with serially diluted Treg in the presence of stimulus. As the Teff to Treg ratio increases, the suppression decreases and modifications of this standardized assay allow for investigation of its different components. In terms of cell function, both *in vitro* and *in vivo* experiments show that activation of nTreg is TCR dependent (Thornton and Shevach, 1998, Walker et al., 2003) and once activated, they have a potential to suppress effector T cells in an antigen non-specific fashion (Thornton and Shevach, 2000).

Investigations into the TCR repertoire of nTreg in a steady state have shown that TCR of nTreg and conventional CD25⁻ T cells are similar in diversity, but distinct and partially overlapping (Hsieh et al., 2006, Wong et al., 2007, Pacholczyk et al., 2007). Proliferation within lymphopenic hosts of conventional T cells transduced with nTreg-derived TCR suggested that the TCR repertoire of nTreg is skewed towards self-reactive when compared to the one of conventional T cells (Hsieh et al., 2006). Other studies, however, demonstrated that that TCR of nTreg can recognize foreign antigens similarly to conventional T cells (Pacholczyk et al., 2007).

1.4.2.1.3. Cell surface markers

CD25 is the high affinity α -chain of the IL-2 receptor and was originally used as a marker to differentiate nTreg (Sakaguchi et al., 1995). It also suggested the importance of IL-2 signalling in the development and/or suppressive function of nTreg. However, as CD25 is upregulated on activated conventional T cells, other markers expressed by nTreg were needed to be identified.

Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), a negative co-stimulator of T cell activation, was shown to be constitutively expressed on naïve CD25⁺ CD4⁺ T cells and its *in vivo* blockade was demonstrated to lead to the development of spontaneous organ-specific autoimmunity (Takahashi et al., 2000). CTLA-4 expression was also found to be critical for Treg-mediated regulation of intestinal inflammation (Read et al., 2000) and type 1 diabetes (Luhder et al., 1998). Finally, CTLA-4 was suggested to be the core mechanism through which Treg control the function of APC by, for example, inhibiting their maturation and in this way suppressing T cell activation (Onishi et al., 2008, Wing et al., 2008, Wing and Sakaguchi, 2010).

Gene expression analysis of naïve CD4⁺ CD25⁺ T cells, in addition to CTLA-4, also revealed increased expression of glucocorticoid-induced TNF receptor-related protein (GITR) (McHugh et al., 2002). GITR was found to be upregulated upon Treg activation and its stimulation via monoclonal antibody abrogated *in vitro* Treg-mediated suppression (McHugh et al., 2002) and *in vivo* induced development of gastritis (Shimizu et al., 2002). It has been suggested that GITR, unlike CTLA-4, might act as a switch-off mechanism for Treg function (Ermann and Fathman, 2003). This was suggested to have potentially beneficial effects by enhancing responses to infections and tumours (Nocentini and Riccardi, 2009).

1.4.2.1.4. Foxp3 expression

The breakthrough in nTreg biology came with the discovery of Foxp3, a master transcription factor driving nTreg lineage (Hori et al., 2003, Fontenot et al., 2003).

Originally a mutation in the *foxp3* gene was identified as the cause of the phenotype observed in scurfy mice which is manifested by multi-organ autoimmunity (Brunkow et al., 2001). The mutation in the X-linked *foxp3* gene leads to splenomegaly, enlarged lymph nodes, and immune cell infiltration of major organs (Godfrey et al., 1991). Increased T cell activation/proliferation due to hyper-responsive TCR and lower co-stimulatory threshold for T cell activation were also reported, all suggestive of an impaired regulatory mechanism (Clark et al., 1999). The phenotype of scurfy mice is very similar to the human disease immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome, which was found to be caused by the mutation in the *foxp3* gene as well (Bennett et al., 2001).

The importance of Foxp3 expression in nTreg was further highlighted by the retroviral transduction of Foxp3 into naïve $CD4^+CD25^-$ T cells which conferred a suppressive phenotype on the naïve cells (Hori et al., 2003, Fontenot et al., 2003). Foxp3-transduced naïve T cells effectively suppressed inflammatory bowel disease and autoimmune gastritis (Hori et al., 2003). Reciprocal experiments with ablated Foxp3 expression in nTreg showed that, unless Foxp3 expression is maintained, nTreg lose their suppressive potential and acquire an ability to extensively proliferate and produce Th1-associated cytokines (Williams and Rudensky, 2007).

Generation of a Foxp3-GFP mouse by Rudensky lab which has a knock-in allele of green-fluorescent protein (GFP) fused to the *foxp3* gene provided a unique tool for easy identification and isolation of nTreg (Fontenot et al., 2005b). The phenotypic analysis of steady-state Foxp3⁺ CD4⁺ cells done in Foxp3-GFP mice revealed that majority (>97%) are CD4⁺, they include both CD25⁺ and CD25⁻ cells, and they express CTLA-4 and GITR as shown before for CD25⁺ cells only (Fontenot et al., 2005b).

Foxp3 was shown to form a DNA-binding complex with nuclear factor of activated T cells (NFAT) to regulate transcription of ~1,100 target genes (Wu et al., 2006, Marson et al., 2007). Among the Foxp3 target genes which are positively regulated are *cd25*, *gitr* and programmed cell death-1 (*pd-1*) (Marson et al., 2007, Zheng et al.,

2007). Interestingly, Foxp3 seems to predominantly suppress transcription of genes involved in T cell activation and function such as *il-2* by occupying their promoter regions (Marson et al., 2007, Zheng et al., 2007).

nTreg have been demonstrated to play an important role in suppressing and promoting resolution of inflammation in autoimmune and allergic disease settings (Tang et al., 2004, McGeachy et al., 2005, Leech et al., 2007). They have been also reported to dampen anti-tumour responses and hence contribute to tumour resistance (Casares et al., 2003, Clarke et al., 2006). Although the knowledge about nTreg is exponentially increasing, the exact mechanisms of how they suppress different forms of inflammation *in vivo* are still elusive and a more definitive understanding of how nTreg function is critical for their effective manipulation. The general suppressive mechanisms have been suggested and are described in section 1.6.

1.4.2.2. Induced Foxp3⁺ Treg

It has been demonstrated that Foxp3 can be induced *de novo* in naïve CD4⁺ T cells under certain conditions both *in vivo* in a periphery and *in vitro*, and these cells have been called induced Treg.

1.4.2.2.1. Discovery of iTreg

One of the first reports on the *de novo* generation of CD4⁺ CD25⁺ regulatory T cells came from the study which used transferred antigen-specific T cells on a RAG2^{-/-} background and demonstrated the appearance of CD25⁺ cells after low dose antigen administration intravenously or orally (Thorstenson and Khoruts, 2001). These CD4⁺ CD25⁺ cells established peripheral tolerance, had a low proliferation rate, were unable to produce IL-2 and had a suppressive potential, all characteristic of regulatory T cell phenotype. The generation of CD4⁺ CD25⁺ cells did not occur following antigen administration in adjuvant (Thorstenson and Khoruts, 2001) suggesting that antigen presentation by immature APC played a role in tolerance induction. Later studies on peripheral tolerance confirmed this phenomenon and

moreover showed that these CD4⁺ CD25⁺ T cells express FoxP3 and are IL-2-dependent (Apostolou and von Boehmer, 2004, Knoechel et al., 2005).

1.4.2.2.2. Importance of TGF- β signalling

In addition to IL-2 signalling demonstrated to be essential for Treg peripheral homeostasis (Knoechel et al., 2005, Fontenot et al., 2005a), transforming growth factor- β (TGF- β) was shown to be required for the generation of induced Treg, both *in vivo* and *in vitro*. TGF- β is a protein that can exist in a secreted and membrane-bound form, and among its diverse functions (Shi and Massague, 2003), the membrane-bound form of TGF- β was found to be involved in a contact-dependent immunosuppression (Nakamura et al., 2001). TGF- β in the presence of TCR signalling was reported to induce Foxp3 expression in naïve CD4⁺ CD25⁻ T cells *in vitro*, and these induced Foxp3⁺ cells expressed membrane-bound TGF- β and suppressed conventional T cells (Chen et al., 2003). TGF- β -converted antigen-specific CD4⁺ CD25⁺ cells also suppressed T cell expansion *in vivo* and polyclonal iTreg were found to ameliorate lung inflammation in a house dust mite (HDM)-induced murine model of asthma (Chen et al., 2003). This phenomenon was confirmed by other studies which also showed that *in vitro* pre-treatment of naïve CD4⁺ CD25⁻ T cells with TGF- β promoted their conversion into Treg under tolerogenic conditions *in vivo* (Kretschmer et al., 2005).

1.4.2.2.3. iTreg versus nTreg

Since the discovery of peripherally-induced Treg, large amount of work has gone into investigating methods of differentiating between natural thymically-derived nTreg and *de novo* generated iTreg. Initially reported to be nTreg-specific, expression of Helios (Thornton et al., 2010) was found to occur in activated T cells and iTreg as well (Akimova et al., 2011, Gottschalk et al., 2012). Interestingly, it has been observed that nTreg have a stably demethylated conserved region of the *foxp3* gene, whereas iTreg do not (Floess et al., 2007). Epigenetic modification of the *foxp3* locus was suggested to be required for stable Foxp3 expression and maintenance of

the suppressive phenotype, as iTreg were found to rapidly lose Foxp3 expression upon re-stimulation in the absence of TGF- β (Floess et al., 2007).

Rapid loss of Foxp3 in re-stimulated iTreg led to the concerns over their potential to convert into effector T cells and hence contribute to the inflammation when used in a therapeutic setting. However, it was shown that ex-iTreg do not acquire pathogenic phenotype and they can effectively suppress T cells *in vitro* and *in vivo* (O'Connor et al., 2010).

To sum up, it has been speculated that the driving force behind iTreg generation in the periphery is the need to maintain mucosal tolerance in the gut and the lung to innocuous antigens, whereas the primary function of nTreg is to control autoimmunity and promote resolution during inflammation (Curotto de Lafaille and Lafaille, 2009).

1.4.2.3. Tr1 and Th3 cells

Another regulatory cell type that was proposed to contribute to peripheral tolerance is T regulatory cell 1 (Tr1). These cells are characterized by the production of large amounts of IL-10 and TGF- β and low levels of IFN- γ and IL-2 (Groux et al., 1997). Similar to nTreg, they have very low proliferative capacity *in vitro* and can effectively suppress antigen-specific T cells *in vitro* and *in vivo* in a model of colitis (Groux et al., 1997). Tr1 cells can be induced from naïve CD4⁺ CD25⁻ T cells in the presence of IL-10, however they do not acquire expression of Foxp3 (Groux et al., 1997, Vieira et al., 2004). Immature DC have been implicated in the induction of Tr1 cells *in vivo* (Jonuleit et al., 2000). Antigen-reactive Tr1 cells have to be activated through their TCR to suppresses T cells, however, once activated they can mediate bystander suppression via local release of anti-inflammatory IL-10 and TGF- β (Groux, 2003, Roncarolo et al., 2006).

Another type of regulatory T cells called Th3 has been initially described in the context of oral tolerance. Th3 cells were reported to provide help to B cells for IgA

production and to produce large amounts of TGF- β (Weiner, 2001). Th3-derived TGF- β was shown to promote *in vivo* generation of Foxp3⁺ iTreg (Carrier et al., 2007), thus suggesting a role for Th3 cells in peripheral tolerance.

1.4.2.4. Other regulatory cell types

Suppressor cells expressing CD8 were originally discovered by Gershon and Kondo in the 1970s (Gershon and Kondo, 1970, Gershon and Kondo, 1971). Difficulties in the characterization of these cells due to the lack of cell-specific marker and the discovery of CD4⁺ CD25⁺ Treg resulted in CD8⁺ regulatory cells (CD8⁺ Treg) being largely abandoned until recent rediscovery. CD8⁺ Treg were reported to be generated *in vivo* after immunization with OVA and they required IFN- γ to mediate TGF- β -dependent suppression of CD4⁺ T cells responses (Myers et al., 2005). This suggested a means by which pathogenic Th1 cells could 'self-regulate' themselves. CD8⁺ Treg were also identified in multiple sclerosis (MS) patients where they showed cytotoxic activity against myelin-reactive CD4⁺ T cells (Correale and Villa, 2008), and have been implicated in the recovery phase in the murine model of MS, experimental autoimmune encephalomyelitis (EAE) (Lee et al., 2008). CD8⁺ Treg are very diverse and some subsets were shown to express Foxp3 (Xystrakis et al., 2004, Bienvenu et al., 2005).

The role of B cells in potentiating immune responses in infections and autoimmune disease has been well established. However, more recently, it was suggested that they can have a regulatory role as well. B cells were shown to be capable of expanding Foxp3⁺ Treg and dictating their antigen-reactivity (Chen et al., 2009), and they were also reported to induce Foxp3⁺ iTreg from CD4⁺ CD25⁻ T cells (Tu et al., 2008). B cell-derived IL-10 was demonstrated to mediate Treg expansion (Sun et al., 2008) and to have a potential in regulating autoimmune pathology (Fillatreau et al., 2002, Mauri et al., 2003).

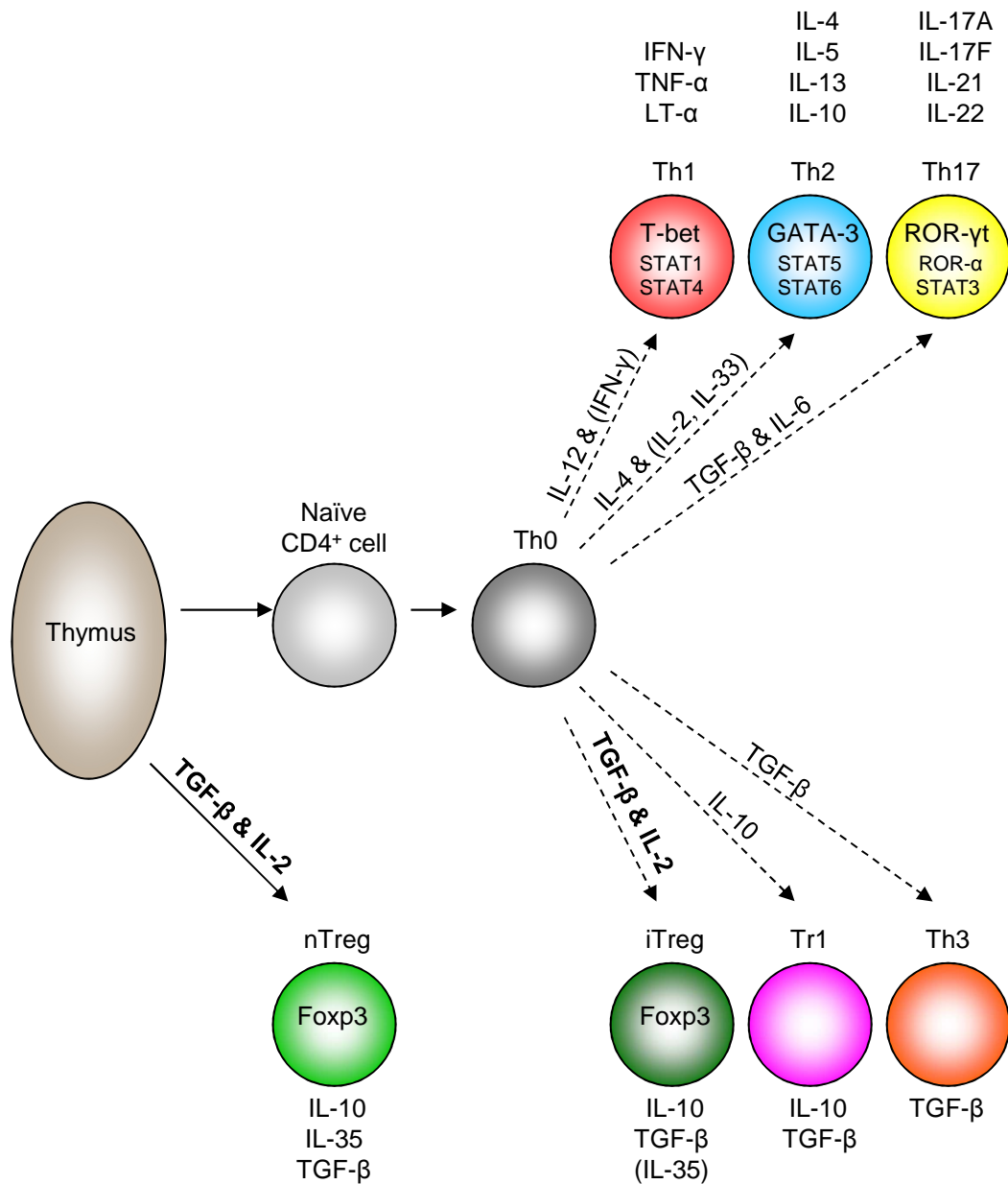


Figure 1.2 Key CD4⁺ T cell subsets.

CD4⁺ T cells exist in the thymus as naïve CD4⁺ CD25⁻ cells or CD4⁺ CD25⁺ nTreg. Upon activation, CD4⁺ T cells (Th0) can differentiate into different effector (Th1, Th2 or Th17) or regulatory subsets (iTreg, Tr1 or Th3) depending on the APC-derived cytokine milieu. Cytokines required for Th cell differentiation and associated transcription factors are shown. In addition, signature cytokines of each subset are listed. Adapted from McPherson, 2012.

1.5. CD4⁺ T cell plasticity

The concept of distinct CD4⁺ T cell lineages has been very useful for understanding how different CD4⁺ T cells and their signature cytokines are driving and controlling different immune responses. However, recent evidence on the flexibility of CD4⁺ T cell lineages has led to the major rethinking of this paradigm (O'Shea and Paul, 2010).

1.5.1. Plasticity of cytokine production

IL-9 previously designated as a signature cytokine for a still to be confirmed Th9 subtype was demonstrated to be produced by both Th2 cells via TGF- β signalling (Veldhoen et al., 2008) and Th17 cells (Elyaman et al., 2009). Th17 cells were reported to acquire an ability to produce a Th1-associated cytokine by either becoming double producers of IL-17 and IFN- γ under certain inflammatory conditions (Wilson et al., 2007, Kebir et al., 2009), or by completely abrogating production of IL-17 and committing to IFN- γ secretion (Lee et al., 2009, Shi et al., 2008). Interestingly, Th1 cells were never demonstrated to produce IL-17, however they were shown to be capable of secreting Th2-associated cytokine IL-13 and to promote airway hyper-responsiveness in a murine model of asthma (Sugimoto et al., 2004). A Th2-dominated environment during helminth infection was observed to convert Th1 and Th17 cells into Th2 effectors secreting IL-4, which are more efficient at tackling extracellular parasites (Panzer et al., 2012). Conversely, IL-4 producing Th2 cells were reported to switch to IFN- γ production after transfer into a model of Th1-skewing infection with lymphocytic choriomeningitis virus (LCMV) (Hegazy et al., 2010).

1.5.2. Flexibility in expression of master transcription factors

CD4⁺ T cell lineages are defined by the specific expression of master transcription factors and expression of these has been found to be not as fixed as it was thought initially. As mentioned above, Hegazy et al., has shown that transfer of Th2 cells into a model of Th1-associated infection with LCMV resulted in the Th2 cells not only

secreting IFN- γ but also stably co-expressing GATA-3 and T-bet (Hegazy et al., 2010). This 'Th1+2' phenotype required TCR stimulation, type I and type II interferons and IL-12 signalling which induced T-bet expression and hence switched on IFN- γ production. Th2 'reprogramming' was suggested to be critical for the control and clearance of LCMV infection. Another example of flexibility in expressing master transcription factors is provided by Th17 cells which were shown to co-express ROR- γ t and T-bet in a model of hen egg lysozyme (HEL)-driven ocular inflammation (Shi et al., 2008). A substantial degree of plasticity has been also reported in Treg lineage and this will be discussed in more detail as it may have important implications in the potential therapeutic use of Treg.

1.5.3. Plasticity of Treg lineage

Suppressive capacity of Treg sparked an interest in their potential use in therapy and among the questions that have been posed was how stable Treg are and whether they have a potential to convert into pathogenic cells. Zhou et al. used a genetic lineage-tracing system in which they could identify not only Foxp3⁺ cells but also cells that expressed Foxp3 at some point in their existence and are now Foxp3⁻, so called exFoxp3 cells (Zhou et al., 2009a). They demonstrated that in a naïve mouse ~20% of total peripheral T cells that once were able to express Foxp3, lost its expression. These exFoxp3 cells had an activated memory cell phenotype and produced IFN- γ and low level of IL-17. Using a spontaneous model of type 1 diabetes, they reported that a pro-inflammatory autoimmune environment promoted loss of Foxp3 and exFoxp3 cells had a substantial pathogenic potential. Interestingly, exFoxp3 cells were shown to be derived both from nTreg and peripheral iTreg. Similar gain of ability to produce pro-inflammatory IL-17 and loss of Foxp3 expression by nTreg, or co-expression of Foxp3 and IFN- γ was reported in other studies (Xu et al., 2007, Yang et al., 2008a, Wei et al., 2009). However, substantial heterogeneity of Treg prompted a question as to whether a particular subset of Treg is more prone to lose Foxp3 and become pathogenic in an inflammatory context (Gadina and O'Shea, 2009).

As mentioned before, Foxp3⁺ cells include both CD25⁺ and CD25⁻ T cells. A study by Komatsu et al. showed that the CD25⁻ fraction, but not CD25⁺, contained a minor population that upon transfer into lymphopenic hosts readily loses Foxp3 (Komatsu et al., 2009). Moreover, CD62L^{high} subpopulation of Treg was demonstrated to be the most effective at suppressing EAE and was shown to retain Foxp3 expression significantly better than its CD62L^{low} counterpart (Stephens et al., 2009). More recently, Treg derived from the inflamed CNS, but not the spleen, during EAE were reported to resist conversion to IL-17-producing cells via IL-6 unresponsiveness indicating that intrinsic mechanisms exist within Treg to stabilize their Foxp3 expression in an inflamed environment (O'Connor et al., 2012).

In conclusion, the flexibility in expression of cytokines and master transcription factors has been suggested to have an evolutionary value as it may allow easier adjustment of immune responses to new pathogens (O'Shea and Paul, 2010). However, plasticity of regulatory cells shows that this possibly important feature of CD4⁺ T cells can be sometimes turned against the host and can contribute to inflammation instead of dampening it. Epigenetic modifications are now suggested to stand behind the regulation of CD4⁺ T cell lineage commitment and the degree of plasticity that occurs within it (Wei et al., 2009).

1.6. Mechanisms of regulation by Treg

Treg have been suggested to mediate suppression via very diverse mechanisms and these were demonstrated to target either T cells directly by suppressor cytokines, IL-2 deprivation and cytolysis, or to target APC (Shevach, 2009). Initial results from *in vitro* studies showed that Treg mediate suppression in a contact-dependent manner and neutralization of IL-10 and TGF- β did not break it (Thornton and Shevach, 1998, Takahashi et al., 1998). However, investigations into regulation *in vivo* soon revealed that Treg behaviour is much more diverse.

1.6.1. Suppressive cytokines

The importance of TGF- β in maintaining immune homeostasis was initially observed in TGF- β 1-deficient mice which showed uncontrollable lymphocyte proliferation and cellular infiltration of major organs (Christ et al., 1994). Multiple studies demonstrated that an important source of TGF- β in the immune system is Treg which expresses high levels both secreted and membrane-bound forms of this cytokine (Nakamura et al., 2001, Schmidt et al., 2012). However, *in vitro* suppression experiments gave contradicting results as to how important TGF- β is in Treg-mediated suppression (Nakamura et al., 2001, Levings et al., 2002, Piccirillo et al., 2002). Investigations *in vivo* in a model of colitis gave unclear results as well. While one study reported that TGF- β is critical in preventing inflammation (Read et al., 2000), a different one demonstrated that its protective role is redundant (Kullberg et al., 2005). Interestingly, the membrane-bound form of TGF- β was implicated in infectious tolerance which can be induced by Treg capable of conferring a suppressive activity on conventional T cells (Andersson et al., 2008).

Another cytokine abundantly produced by Treg is IL-10 and its importance in mediating immunosuppression has been well established. Blocking IL-10 or using IL-10-deficient Treg was shown to abrogate protective effects of Treg in models of colitis (Asseman et al., 1999) and allergic airways inflammation (Leech et al., 2007). Treg-derived IL-10 was also implicated in the natural disease resolution in a model of EAE (McGeachy et al., 2005) and a high level of IL-10 expression within the CNS was shown to inhibit inflammation (Cua et al., 2001). Moreover, it was demonstrated that IL-10 is important in controlling antigen-activated and/or memory T cells but not naïve T cells (Kamanaka et al., 2011) suggesting that mechanisms of suppression may vary according to the activation status of a target cell (Schmidt et al., 2012).

A novel inhibitory cytokine called IL-35, a member of IL-12 family, was reported to be constitutively expressed only by Treg and markedly upregulated upon co-culture of Treg with effector T cells (Collison et al., 2007). Ectopic expression of IL-35 in conventional T cells endowed them with a suppressive capacity. IL-35 was shown to inhibit proliferation of Th17 cells *in vitro* and to suppress a murine collagen-induced

model of arthritis (Niedbala et al., 2007). Its ability to convert effector T cells into IL-35-producing T cells suggested that IL-35 can also contribute to infectious tolerance (Chaturvedi et al., 2011).

1.6.2. Cytolysis

One of the potential mechanisms that could explain reported observations that separation of Treg and effector T cells by a permeable membrane breaks the suppression or that it is unaffected by blocking suppressive cytokines (Thornton and Shevach, 1998, Takahashi et al., 1998) is a contact-dependent suppression via cytolysis (Shevach, 2002). Cytolysis is a mechanism in which cytotoxic molecules such as proteases produced by one cell induce apoptosis of the neighbouring cell. This ‘killing’ mechanism has been well documented in CD8⁺ T cells and natural killer (NK) cells, however it has been unclear if Treg were capable of similar behaviour. Gondek et al. demonstrated that indeed Treg can produce granzyme B, a serine protease, which was observed to induce apoptosis in co-cultured effector T cells in a perforin-independent fashion *in vitro* (Gondek et al., 2005). Granzyme B-deficient Treg were also shown to have reduced suppressive capacity *in vitro* (Gondek et al., 2005), and the relevance of this mechanism was confirmed *in vivo* in studies on tumour resistance (Cao et al., 2007, Boissonnas et al., 2010). Another example of cytotoxic Treg activity is suppression mediated by TNF-related apoptosis-inducing ligand (TRAIL) which induces apoptosis of Teff by binding to a death receptor 5 (DR5) upregulated on Teff upon activation (Ren et al., 2007).

1.6.3. Metabolic disruption

Treg are known to express the high affinity IL-2 receptor α -chain (CD25) and require IL-2 for their survival, growth and function (de la Rosa et al., 2004). However, as they were shown to be incapable of producing IL-2 themselves, they were suggested to act as a ‘sink’ and deprive effector T cells of IL-2 which is critical for their activation, differentiation and proliferation (Sakaguchi et al., 1995). This was demonstrated to be true both *in vitro* and *in vivo* in a model of inflammatory bowel disease (IBD) (de la Rosa et al., 2004, Pandiyan et al., 2007).

1.6.4. Modulation of APC

Conventional T cells have been shown to undergo apoptosis or become anergic if they are stimulated by APC in the absence of co-stimulatory signals (as discussed in section 1.3.2). Hence, one of the Treg-mediated suppressive mechanisms was suggested to act via APC. It was observed *in vitro* that CD4⁺ CD25⁺ T cells were able to down-regulate the expression of co-stimulatory molecules CD80 and CD86 on DC via CTLA-4 (Cederbom et al., 2000, Oderup et al., 2006). Specific CTLA-4 deficiency in Treg was shown to lead to spontaneous development of autoimmune lymphoproliferative disorder in mice (Wing et al., 2008). CTLA-4-deficient Treg were found to lack the capacity to down-regulate CD80 and CD86 expression on DC and hence were unable to limit T cell responses in that model. CTLA-4 ligation of CD80 and/or CD86 was also demonstrated to induce expression of indoleamine 2,3-dioxygenase (IDO) pathway in DC leading to the production of tryptophan catabolites such as kynurenin that were suggested to exert immunosuppressive effects (Grohmann et al., 2002, Fallarino et al., 2003). Moreover, negative stimulation of DC through CTLA-4 was shown to induce nuclear localization of a transcription factor Foxo3 which inhibited production of pro-inflammatory cytokines IL-6 and TNF by DC compromising their stimulatory capacity (Dejean et al., 2009).

A novel mechanism of Treg-mediated suppression of DC function via CTLA-4 was recently reported by the Sansom group who showed that CTLA-4 can remove co-stimulatory CD86 from the cell surface of a DC via trans-endocytosis (Qureshi et al., 2011). They have used a CTLA-4 expressing cell line to demonstrate this *in vitro* and *in vivo* and found that CD86 acquired in such a way was subsequently degraded in endosomal compartments within CTLA-4-expressing cells. This finding was also confirmed to occur in Treg and shown to be antigen-dependent (Qureshi et al., 2011).

Another cell surface inhibitory molecule expressed by Treg is lymphocyte activation gene-3 (LAG-3) which binds to MHC class II on DC and inhibits DC maturation and their co-stimulatory capacity (Liang et al., 2008). Treg can also modulate the array of cytokines produced by DC skewing it towards an anti-inflammatory type which includes IL-10 (Misra et al., 2004, Veldhoen et al., 2006). Moreover, they were

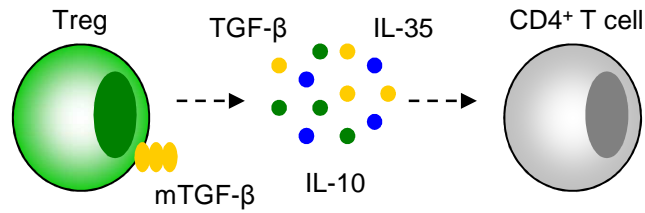
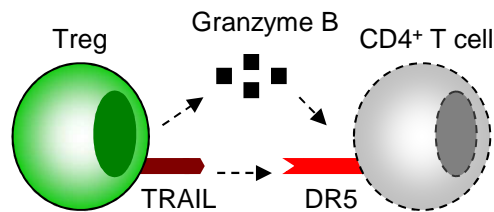
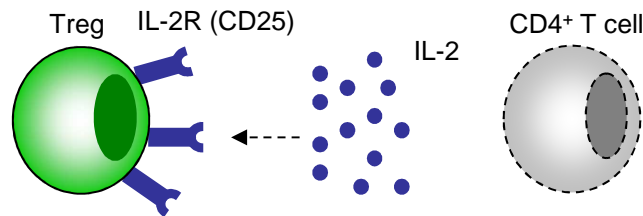
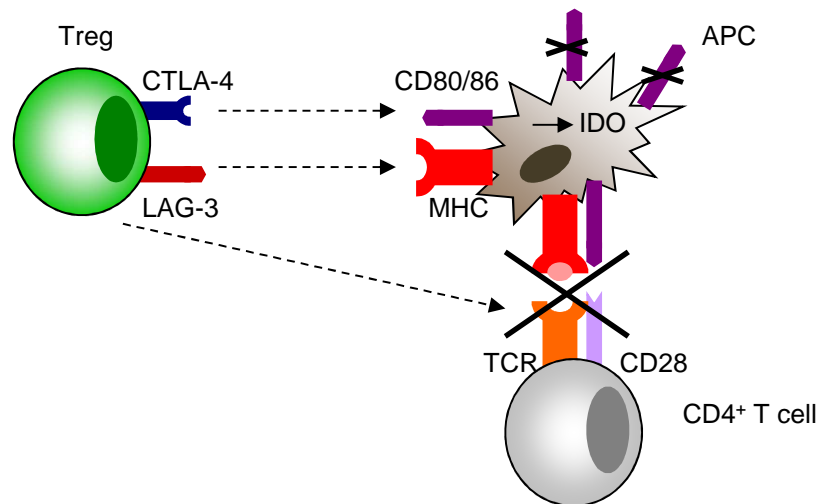
shown to inhibit stable contacts between DC and naïve CD4⁺ T cells *in vivo* during priming which are required for T cell activation (Tadokoro et al., 2006).

1.6.5. Other mechanisms - adenosine

In addition to suppressive cytokines, a different type of anti-inflammatory mediator that was discovered to be produced by Treg is adenosine. Adenosine generation is catalyzed by two membrane-bound ecto-enzymes: CD39 is an ecto-ATP diphosphohydrolase which converts circulating ATP and ADP into 5'AMP and CD73 is an ecto-5'-nucleotidase which converts 5'AMP to adenosine. Adenosine can bind to adenosine receptors expressed on lymphocytes and myeloid cells and can very effectively regulate inflammation via a variety of mechanisms (Linden, 2006). Expression of CD73 on Treg was firstly reported by Kobie et al. who also showed that adenosine produced by Treg suppressed proliferation and cytokine production by Th1 and Th2 cells (Kobie et al., 2006). This was shortly followed by another study demonstrating that Treg can also express CD39 (Deaglio et al., 2007). This coordinated CD39/CD73 expression was suggested to be an important part of Treg suppressive machinery.

Treg can clearly display a wide variety of mechanisms which they use to regulate inflammation. The summary diagram is provided at the end of this section in Figure 1.3. This complexity and possibly cross-regulation allows for the best chances to keep self- and excessive inflammatory responses under control. Moreover, at least some of the mechanisms mentioned above have been demonstrated to be redundant or plastic. Pillai et al. showed that IL-10/IL-35-deficient Treg are fully functional *in vivo* and can still mediate suppression by upregulating cytotoxicity via TRAIL (Pillai et al., 2011).

The very important questions to answer are whether different Treg subsets such as nTreg and iTreg vary in their regulatory mechanisms and whether different mechanisms occur in different types and/or sites of inflammation.

A Production of anti-inflammatory cytokines**B** Cytolysis**C** Metabolic disruption**D** Modulation of APC**Figure 1.3 Mechanisms of Treg-mediated suppression.**

A) Production of anti-inflammatory cytokines. B) Cytolysis via granzyme B or ligation of death receptor-5 (DR5) on CD4⁺ T cell surface by TRAIL. C) Metabolic disruption via IL-2 deprivation. D) Modulation of APC: inhibition of co-stimulatory activity and/or maturation of APC via CTLA-4 and LAG-3 signalling, induction of IDO expression and inhibition of stable APC-T cell contacts required for T cell activation. Dotted line indicates compromised cell viability.

1.7. Regulatory CD4⁺ T cells in autoimmune disease

Autoimmune disease occurs when the central and/or peripheral tolerance is compromised and an inflammatory response directed by the immune cells at self-tissues becomes uncontrollable. Autoimmune diseases can be broadly divided into systemic such as systemic lupus erythematosus or organ-specific such as type 1 diabetes mellitus or rheumatoid arthritis. The causes underlying most autoimmune diseases are unknown and it is speculated that they include a mixture of both genetic and environmental factors (Davidson and Diamond, 2001). A classic example of an autoimmune disease is multiple sclerosis which will be thoroughly discussed in this thesis.

1.7.1. Multiple sclerosis

Multiple sclerosis (MS) is a complex demyelinating autoimmune disease of the central nervous system (CNS) characterized by chronic inflammation. It affects woman more frequently than man, and usually starts between 20 and 40 years of age (Sellner et al., 2011) MS is most prevalent in northern Europe, southern Australia and North America (Kurtzke, 1991). The etiology of MS is still unknown and multiple genetic and environment factors have been implicated (Sospedra and Martin, 2005).

1.7.1.1. Symptoms and clinical courses of MS

MS is a very heterogeneous disease and more accurately it is considered to be a group of neurological disorders rather than one disease. The symptoms vary from one patient to another and are associated with the location of the lesions within the CNS (Charil et al., 2003, Kincses et al., 2010). MS most commonly starts with vision problems due to optic neuritis, numbness sensations and other sensory disturbances, muscle weakness and spasms, problems with balance and coordination, and fatigue (Noseworthy et al., 2000). Cognitive impairment usually appears as the disease progresses. The diagnosis is based on the clinical and laboratory criteria, and usually involves magnetic resonance imaging (MRI) which enables visualisation of the lesions within the CNS.

There are three recognized clinical courses in MS: relapsing-remitting MS (RR-MS), secondary progressive MS (SP-MS) and primary progressive MS (PP-MS). RR-MS is the most frequent and occurs in 85-90% of patients with women affected twice as often as man (Sospedra and Martin, 2005). This form of MS is characterized by periods of symptoms of the disease which are followed by months to years of remission. Gradually the patients have shorter and scarcer periods of remission, and most of them go on to develop SP-MS which means progressive neurodegeneration. An estimated 10-15% of patients display PP-MS, with both genders affected equally, where the disease is characterized by steady disease progression (Noseworthy et al., 2000). It is speculated that disease initiation and relapse phases in RR-MS are associated with active inflammation within the CNS, which eventually leads to irreparable axonal damage and neuronal loss, marking SP-MS (Bruck, 2005).

1.7.1.2. Causes underlying MS

The causes of MS remain unclear and they have been suggested to include both genetic susceptibility and environmental triggers. The genetic contribution was reported in many studies which found that first degree relatives of an MS patient are 15 to 25 times more likely to develop MS relative to the general population (Dyment et al., 2004). This likelihood increases by 150-fold if both parents have MS, and 170-fold if one of the monozygotic female twins is affected (Dyment et al., 2004). The search for susceptibility genes using a genome-wide linkage studies has confirmed significance in the MHC region on the chromosome 6p21 (Games and Cooperative, 2003) which was first suggested in 1970s. The specific genes that were found to confer risk of MS are HLA-DR and –DQ genes, and the susceptibility haplotypes include HLA-DR15, HLA-DR2 and HLA-DQ6 (Olerup and Hillert, 1991, Barcellos et al., 2003). How MHC class II genes confer susceptibility to MS at the molecular level is unclear, however it is known that they encode antigen presentation machinery which is critical to T cell activation. Among the suggested mechanisms are the preference for presentation of self-peptides and escaping negative selection through binding only selective peptides (Sospedra and Martin, 2005). Multiple genome-wide association studies (GWAS) also identified MS-associated single

nucleotide polymorphisms (SNPs) that are located outside the MHC region and are within the genes encoding CD25, IL-7R α chain (Hafler et al., 2007), CD40 and CYP27B1 (Consortium, 2009) which is an enzyme generating an active form of vitamin D implicated in MS as discussed below.

The fact that key susceptibility genes in MS encode components of the immune system strongly supports the idea that MS is an autoimmune disease and implicates autoreactive CD4⁺ T cells in driving its pathology.

In addition to genetic links, the etiology of MS is thought to have environmental contributions as well. There is an observed increase in MS prevalence with increasing distance north or south of the equator and migration from the area of high prevalence to the area of low prevalence, at the age of less than 15-16, was reported to lower the risk of disease (Kurtzke, 1991). The latitudinal distribution of MS prevalence has been associated with decreased sun exposure indicating that sunlight is a protective factor in MS. Ultraviolet B radiation from sunlight induces synthesis of the precursor of vitamin D in the skin and has been first linked to MS by Goldberg in 1974 (Hayes, 2000). Some studies have indicated that intake of vitamin D may lower the risk of developing MS (Munger et al., 2004) and, some but not all, also reported on the lower serum levels of vitamin D in the blood of MS patients (Soilu-Hanninen et al., 2005). Protective effects of vitamin D were also demonstrated in the animal model of MS, experimental autoimmune encephalomyelitis (EAE). Calcitriol, an active metabolite of vitamin D₃, has been shown to suppress both the induction and progression of EAE and this is thought to occur via inhibition of Th1 responses and promotion of IL-10 signalling (Mattner et al., 2000, Spach and Hayes, 2005, Spach et al., 2006).

A different environmental trigger implicated in MS is a viral infection. As discussed in section 1.3.2, self-reactive T cells that escape negative selection can be activated in the periphery in the presence of danger signals, for example via molecular mimicry or bystander activation. Several viruses have been linked to MS and these include Epstein-Barr virus (EBV) and human herpes virus-6 (HHV-6) (Ascherio et

al., 2001, Soldan et al., 1997), and potential molecular mimics for the CNS self-peptide, myelin basic protein (MBP), have been identified in both species (Challoner et al., 1995, Holmoy et al., 2004). The involvement of an infectious trigger in MS is supported by animal studies which show that TCR-transgenic mice specific for MBP which are housed in non-sterile conditions develop spontaneous EAE, whereas those housed in a pathogen-free environment are disease free (Goverman et al., 1993).

It is very likely that such a heterogeneous disease as MS has multiple genetic and environmental links which interact with each other and the underlying causes vary among the patients as do the symptoms and immunopathology.

1.7.1.3. Immunopathology

It is believed that multiple sclerosis starts with the formation of acute inflammatory lesions as a result of a breakdown of the blood-brain-barrier (BBB) (McFarland and Martin, 2007). The BBB is made of cerebral endothelial cells and is impermeable to solutes and cells only selectively allowing the entrance of essential nutrients. The trigger for BBB breakdown is unknown, however, it is speculated to involve the effect of cytokines which could be produced in response to ongoing infection (Minagar and Alexander, 2003). The reasons for the initiation of the immune response remain unidentifiable as well but it is suspected to target self-peptides found within the CNS. Among the putative antigens are components of myelin protein such as myelin oligodendrocyte glycoprotein (MOG), myelin basic protein (MBP) and proteolipid protein (PLP) (Sospedra and Martin, 2005). The immune responses implicated in immunopathology in MS are summarised in Figure 1.4. at the end of this section.

1.7.1.3.1. CD4⁺ T cell-driven inflammation

Inflammatory reaction in MS is thought to be driven by self-reactive CD4⁺ T cells which are activated by APC in the periphery and are able to cross BBB into the CNS to orchestrate immune response directed at myelin sheath (Ransohoff et al., 2003,

Goverman, 2009). This is supported by the fact that CD4⁺ T cells detected in the peripheral blood and cerebrospinal fluid (CSF) of MS patients were found to be reactive to MBP and PLP, and were contained mostly within the memory/activated T cell population (Illes et al., 1999, Burns et al., 1999, Bielekova et al., 2004). Originally it was considered that Th1 cells drive CNS inflammation as increased IL-12 and IFN- γ levels were reported in serum and CSF of MS patients (Nicoletti et al., 1996, Drulovic et al., 1997, Gutcher and Becher, 2007), and IFN- γ given to MS patients led to the substantial disease deterioration (Panitch et al., 1987). Discovery of Th17 cells and detection of IL-17⁺ T cells infiltrating the CNS of MS patients (Tzartos et al., 2008) led to the conclusion that both Th1 and Th17 cells are implicated in driving inflammation. This is further supported by observations made from the animal model of MS, EAE, which will be discussed in more detail later in this chapter.

Once CD4⁺ T cells gain access to the CNS, they are thought to be reactivated by CNS resident APC such as microglia and start producing pro-inflammatory cytokines that activate local APC and recruit other immune cells (Benveniste, 1997, Goverman, 2009). In addition to autoreactive CD4⁺ T cells, CD8⁺ T cells, B cells and myelin-specific antibodies have been also detected in inflammatory lesions of MS patients (Esiri, 1977, Genain et al., 1999, Friese and Fugger, 2005) and hence are implicated in MS pathology. The inflammatory reaction targeted at the myelin sheath results in its damage and loss, and this demyelination causes axonal death and eventually neurodegeneration. It is known that axons can be remyelinated by oligodendrocyte precursor cells under certain circumstances, however this process seems to fail in MS and the reasons for this are unknown (Franklin and Ffrench-Constant, 2008).

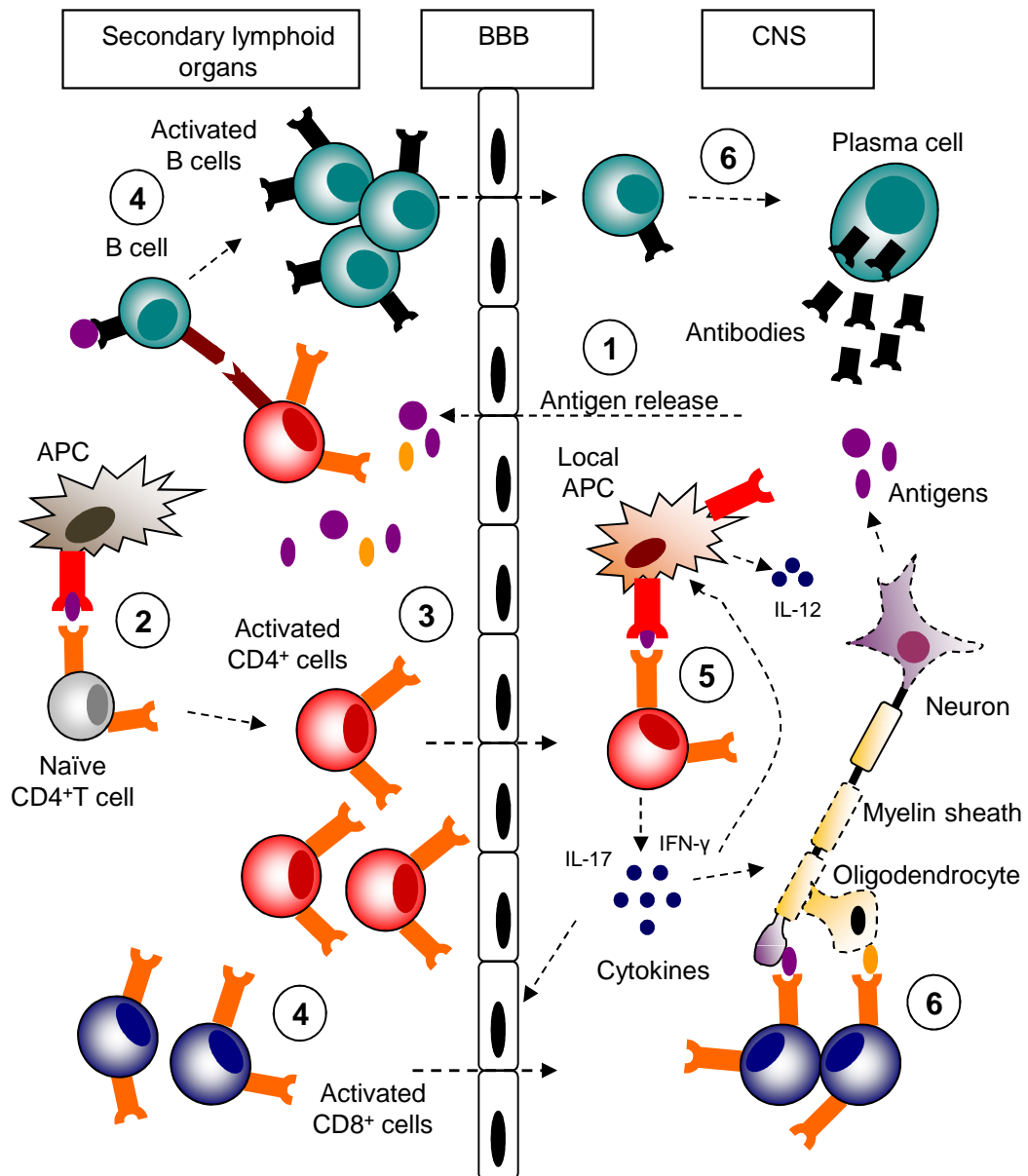


Figure 1.4 Immune responses in MS.

Hypothetical view of immune responses in acute MS lesions. (1) Unknown trigger leads to the release of CNS-derived antigens which (2) are taken up by APC and presented to T cells and B cells in secondary lymphoid organs. Alternatively, autoreactive T cells and B cells may become activated during, for example, on-going infection via molecular mimicry. (3) Activated CD4⁺ cells proliferate, (4) provide help for B cells and CD8⁺ cells and traffic into the CNS. (5) Within the CNS, CD4⁺ cells become re-activated by local APC such as microglia and orchestrate an inflammatory reaction targeted at myelin sheath by producing pro-inflammatory cytokines such as IFN-γ and IL-17. These increase the permeability of BBB, recruit more immune cells into the CNS and promote neuronal demyelination. (6) Following migration into the CNS, activated CD8⁺ cells destroy neuronal cells via cytotoxicity and activated B cells become plasma cells that produce antibodies directed at self-antigens. The CD4⁺ cells thought to drive the inflammatory reaction are Th1 and Th17 cells. Dotted line indicates compromised myelin sheath/cell viability. Adapted from Hemmer et al., 2002.

1.7.1.3.2. Impaired Treg regulation

The breakdown of self-tolerance in MS and hence the disease pathology is thought to be caused not only by pro-inflammatory responses but also by compromised regulatory mechanisms. As described in section 1.4.2, CD4⁺ regulatory T cells are critical to peripheral tolerance by their ability to suppress T cells. Viglietta et al. have reported that although the frequency of peripheral Treg is similar in relapsing-remitting (RR)-MS patients and healthy controls, Treg function is significantly impaired (Viglietta et al., 2004). The incapability to generate Treg clones from MS patients led to the suggestion of a possible clonal exhaustion of Treg *in vivo* (Viglietta et al., 2004). Another study extended those results by showing that Treg from healthy donors suppress MOG-stimulated T cells, whereas Treg from RR-MS patients do not, and this is most likely related to the intrinsic defect in Treg regulatory mechanism and not Treg survival or resistance of responder cells (Haas et al., 2005). Comparisons of peripheral Treg from RR-MS and secondary progressive (SP)-MS patients confirmed unchanged Treg frequencies in both groups of MS patients and found that SP-MS patients had normal Treg function compared to RR-MS patients (Venken et al., 2006). Treg from SP-MS patients effectively suppressed T cell proliferation and IFN- γ production, and had normal levels of Foxp3 expression. Investigations into more disease-relevant Treg revealed that Treg frequencies in the blood and CSF of RR-MS patients are comparable, however the function of CSF-derived Treg has not been tested due to insufficient cell numbers (Haas et al., 2005).

Reports on Treg in the inflamed CNS itself are controversial. As it is impossible to extract CNS cells from the patients, the only studies that can be done are immunohistological analyses of post-mortem MS brain. Tzartos et al. have found no evidence of the presence of Foxp3⁺ cells in the MS lesions (Tzartos et al., 2008). However, another study has reported detectable but very low Foxp3⁺ Treg numbers in RR-MS active brain lesions (Fritzsche et al., 2011). Moreover, as the numbers of apoptosis-prone Treg from CSF were reported to be higher than their numbers from the peripheral blood of the corresponding patients, Fritzsche et al. suggested a model of intracerebral elimination of Treg via CD95L-mediated apoptosis.

As most studies investigate the function of peripheral Treg, an important question to ask is how well these reflect the disease-relevant CNS-derived Treg (O'Connor and Anderton, 2008). Observations made from the animal models of MS suggested that peripheral and CNS-derived Treg are two very different cell populations.

1.7.1.4. Current treatments

All of the current treatments for MS target the immune system and aim to modulate it in a non-specific way. The main ones include interferon- β , glatiramer acetate, mitoxantrone and natalizumab (Berger, 2009).

Interferon- β therapeutics (Avonex®, Rebif®, Betaseron®, Extavia®) are clinically approved for the treatment of RR-MS and include both interferon- β 1a and interferon- β 1b. They have been shown to reduce the frequency of relapses (Jacobs et al., 1996, Kappos, 2002) and although the mode of action remains unclear, they are thought to shift the cytokine production away from pro-inflammatory cytokines and repress T cell responses (Ozenci et al., 2000, Markowitz, 2007).

Glatiramer acetate (Copaxone®) is a synthetic amino acid polymer also approved for the treatment of RR-MS. It was originally developed to mimic CNS self-antigen MBP to induce an animal model of MS, EAE, but was found to inhibit it (Teitelbaum et al., 1971). Initially thought to mainly displace autoantigenic peptides from MHC class II-binding grooves (Aharoni et al., 1999), it was also shown to interfere with DC differentiation (Weber et al., 2004) and shift the T cell response towards Th2 type (Duda et al., 2000).

Mitoxantrone (Novantrone®) is an immunosuppressive cytotoxic drug mostly prescribed to treat SP-MS and aggressive forms of RR-MS in which interferon- β and glatiramer acetate treatment fails (Correale et al., 2005). It intercalates with DNA leading to DNA damage and death of actively proliferating cells such as T cells, B cells and macrophages (Fidler et al., 1986, Fox, 2006). It has been shown to slow

down disease progression in SP-MS and extend the time between relapses in RR-MS (Hartung et al., 2002, Fox, 2006).

Natalizumab (Tysabri®) is a humanized monoclonal antibody directed against the cell adhesion molecule α 4-integrin and was shown to inhibit the entry of lymphocytes into the CNS (Engelhardt and Kappos, 2008). Natalizumab was reported to slow down the disease progression and the rate of relapses in RR-MS (Polman et al., 2006, Hutchinson, 2007). It was briefly removed from the market in 2005 following deaths of several patients who developed progressive multi-focal encephalopathy (PML), which is an opportunistic infection caused by reactivation of JC virus and leads to CNS demyelination (Langer-Gould et al., 2005). The drug was re-introduced and rare cases of PML are still being reported.

Other novel treatments include fingolimod (FTY 710, Gilenya®), alemtuzumab (Campath®) and rituximab (Rituxan®). Fingolimod is a sphingosine 1-phosphate receptor modulator that sequesters lymphocytes in lymph nodes preventing them from trafficking into the CNS (Brinkmann et al., 2010). Alemtuzumab and rituximab are both monoclonal antibodies which target CD52 expressed on the surface of mature lymphocytes (Minagar et al., 2010) and CD20 expressed on B cells, respectively (Hauser et al., 2008).

All of the current MS treatments target the immune system in a non-specific way leaving the patients substantially immunocompromised and prone to infections. The need to target pathogenic T cells specifically or to promote regulatory mechanisms is unquestionable and the animal models of MS provide a promising tool for the investigation of potential therapeutic agents.

1.7.2. EAE as a model of MS

Experimental autoimmune encephalomyelitis (EAE) is an animal model of autoimmune CNS inflammation which shares many histopathological similarities with MS. The origins of the EAE model date back to 1885 when Louis Pasteur

developed a vaccine against rabies which consisted of emulsions of pre-dried spinal cords of rabbits infected with the virus (Baxter, 2007). Reported cases of paralysis led to more thorough investigations which identified cellular infiltrates in the brain and demyelination, both characteristic of encephalomyelitis (Baxter, 2007). Since then, the model has been replicated in a variety of animal species and improved to include one injection of CNS homogenate or a myelin component in a complete Freund's adjuvant (CFA) which is a mineral oil emulsion containing heat-inactivated mycobacterium.

1.7.2.1. Immunopathology

EAE can be induced in susceptible mouse strains by immunization with the whole protein or encephalitogenic peptides from the CNS-derived MBP, MOG or PLP (Zamvil et al., 1986, Mendel et al., 1995, Greer et al., 1992). The disease can be also transferred by CD4⁺ T cells isolated from immunised wild-type (WT) mice or by *in vitro* activated TCR transgenic T cells injected into naïve WT hosts which supports the fact that EAE is a CD4⁺ T cell-driven disease (Zamvil et al., 1985, Stromnes and Goverman, 2006).

The importance of CD4⁺ T cells in initiating and mediating EAE was also confirmed by cell depletion experiments in which monoclonal antibody directed against CD4⁺ T cell marker prevented and reversed EAE when given to paralysed mice (Waldor et al., 1985, Abdul-Majid et al., 2003). The role of CD8⁺ T cells in driving EAE pathology is somewhat controversial. Some studies demonstrated that CD8⁺ T cell depletion does not affect the disease (Abdul-Majid et al., 2003) and CD8⁺ T cells themselves are not able to transfer disease in a MOG-driven EAE model (Leech et al., 2011). Others have shown that CD8⁺ T cells have pathogenic potential and can induce EAE in both MOG- and MBP-driven models (Huseby et al., 2001, Sun et al., 2001). As antibodies against MBP and MOG can be detected in EAE and have been shown to contribute to disease pathology (Iglesias et al., 2001), B cells were also implicated in the disease initiation. B cell-depleted mice were found to have a similar time of EAE onset and overall disease severity as WT mice, however their recovery

time was prolonged (Wolf et al., 1996). This suggested that whereas B cells do not have a role in the activation of pathogenic T cells, they might contribute to the disease regulation. In support of this, B cells have been later demonstrated to contribute to EAE recovery via IL-10 production (Fillatreau et al., 2002).

In order to traffic into the CNS and initiate inflammation, CD4⁺ T cells have to be first activated in the periphery by APC (Goverman, 2009). In EAE, CD4⁺ T cells are either activated by antigen in CFA in lymph nodes draining the site of immunization (Furtado et al., 2008) or they are injected intravenously after being activated *in vitro*. Once CD4⁺ T cells enter the CNS, they are re-activated by CNS-resident macrophages and DC and start producing pro-inflammatory cytokines (Greter et al., 2005). These activate CNS-resident microglia (Ponomarev et al., 2005), and recruit more immune cells into the CNS such as peripheral macrophages which have been shown to be important for initiating inflammation in EAE (Tran et al., 1998). Activated microglia upregulate MHC class II expression and co-stimulatory molecules and become competent APC (Ponomarev et al., 2005). They also start producing pro-inflammatory cytokines such as IL-1, IL-6, TNF- α (Bauer et al., 1993, Renno et al., 1995), reactive oxygen species and nitric oxide synthase (Ruuls et al., 1995, Tran et al., 1997) contributing to further disruption of BBB and cell recruitment into the CNS (Prendergast and Anderton, 2009). Pro-inflammatory mediators produced by activated T cells, macrophages and microglia lead to demyelination and axonal death (Benveniste, 1997, Goverman, 2009).

It is still unclear how CD4⁺ T cells activated in the periphery gain entry into the CNS. Several routes of entry and chemokine receptors and adhesion molecules that facilitate this process have been implicated (Prendergast and Anderton, 2009). Monoclonal antibody to $\alpha 4\beta 1$ -integrin, which mediates attachment and rolling of activated T cells and macrophages to the inflamed brain epithelium, was found to inhibit EAE (Yednock et al., 1992). This has led to successful translation into therapy as an effective drug natalizumab (Rice et al., 2005). A chemokine receptor CCR6 has been found to be expressed by a subset of pathogenic Th17 cells and shown to facilitate their entry into the CNS via choroid plexus (Reboldi et al., 2009).

This, however, was questioned by other studies showing that CCR6-deficient mice develop more severe EAE than WT mice (Elhofy et al., 2009, Villares et al., 2009). A chemokine receptor CXCR3 was reported to be over-expressed in CSF of MS patients (Sorensen et al., 1999), and it is known to be preferentially expressed by Th1 cells. However, its role in T cell recruitment into the CNS has been left unclear after demonstration that its blockade can inhibit active model of EAE, but not a passive model (Sporici and Issekutz, 2010).

CD4⁺ T cells driving EAE were originally considered to be Th1 cells (Ando et al., 1989, Bright et al., 1998b). IL-12 mediating Th1 differentiation was found to be expressed in the CNS and lymphoid tissues of mice with EAE (Bright et al., 1998b), and inhibition of IL-12 signalling suppressed EAE (Leonard et al., 1996, Bright et al., 1998a). However, IFN- γ -deficient mice hypothesised to be resistant to EAE displayed a severe form of disease (Ferber et al., 1996). These conflicting results were cleared up by the discovery of IL-23, a heterodimeric cytokine which shares a p40 subunit with IL-12 (Oppmann et al., 2000). Cua et al. generated specific IL-12-deficient (p35^{-/-}) and IL-23-deficient (p19^{-/-}) mice for comparison with p40^{-/-} mice lacking both IL-12 and IL-23 (Cua et al., 2003). It was the p19^{-/-} mice that were found to be resistant to EAE, and not p35^{-/-} mice, highlighting the importance of IL-23 in mediating EAE (Cua et al., 2003). Soon after, IL-23 was demonstrated to drive differentiation of a new Th cell subset, Th17 (Langrish et al., 2005), pointing out the importance of these cells in driving EAE. Since then, however, conflicting evidence as to whether Th17 cells are able to initiate CNS inflammation has been reported (Komiyama et al., 2006, O'Connor et al., 2008).

Currently it is thought that both Th1 and Th17 cells are important in mediating EAE and more recently an attention has been drawn to a different pro-inflammatory cytokine, GM-CSF, produced by both Th1 and Th17 cells (El-Behi et al., 2011), as it was found to be essential for the EAE induction (Codarri et al., 2011).

1.7.2.2. TCR transgenic models of EAE

The use of TCR transgenic mice enables tracing and studying myelin-reactive T cells as their frequency in WT mice is very low. These have been generated by a combination of $V\alpha$ and $V\beta$ genes that were found in encephalitogenic T cell clones made from T cells isolated from mice immunised with a particular CNS autoantigen. Tg4 TCR transgenic mice have $CD4^+$ T cells that specifically recognize the Ac1-9 peptide of MBP (Liu et al., 1995), $CD4^+$ T cells from 2D2 TCR transgenic mice recognize the 35-55 peptide of MOG protein (Bettelli et al., 2003) and $CD4^+$ T cells from 5B6 TCR transgenic mice recognize peptide 139-151 of PLP protein (Waldner et al., 2000).

EAE can be induced as an 'active' EAE' or a 'passive transfer' EAE. Active EAE is induced in susceptible mouse strains upon immunisation with CNS autoantigen, either a whole protein or an encephalitogenic peptide, in CFA. The susceptible strains for MBP include B10.PL or PL/J mice (both $H-2^u$ haplotype), for MOG C57BL/6 mice ($H-2^b$ haplotype) and for PLP SJL ($H-2^s$ haplotype). In active EAE traceable naïve $CD4^+$ T cells isolated from TCR transgenic mice can also be injected to study pathogenic T cells (O'Connor et al., 2010). Passive EAE uses either Th1 polarised MOG_{35-55} -reactive cells isolated from MOG-immunised mice or *in vitro* stimulated and polarised MBP-reactive $CD4^+$ T cells isolated from Tg4 mice (O'Connor et al., 2007, O'Connor et al., 2010).

There are also different models of EAE characterised by differences in clinical disease curves and these include monophasic (self-resolving) EAE, chronic relapsing-remitting EAE and spontaneous EAE, all recapitulating different aspects of MS. These models of EAE depend on the type of CNS autoantigen and the strain of mouse that is used. In this thesis a monophasic EAE is studied, which is induced in C57BL/6 mice by immunisation with MOG_{35-55} -peptide.

1.7.2.3. Regulation of EAE by Treg

Regulatory T cells were demonstrated to be capable of actively regulating CNS inflammation in EAE. One of the first reports used polyclonal CD4⁺ CD25⁺ T cells and showed that they suppressed both active and passive models of EAE (Kohm et al., 2002). Interestingly, the initial priming and activation of pathogenic T cells in draining lymph nodes were unaffected by Treg, however the numbers of IL-2 secreting effector T cells were reduced and the T cell response was skewed towards Th2 (Kohm et al., 2002). Depletion of CD25⁺ cells prior to EAE induction was demonstrated to significantly exacerbate the disease (Zhang et al., 2004). Moreover, IL-10-deficient CD4⁺ CD25⁺ T cells were less efficient at suppressing EAE which suggested a natural role for Treg-derived IL-10 in controlling the CNS inflammation (Zhang et al., 2004).

Treg were also shown to mediate natural recovery in a monophasic MOG₃₅₋₅₅-driven EAE model (McGeachy et al., 2005). The recovery was associated with an accumulation of IL-10-secreting Foxp3⁺ Treg in the CNS which constituted approximately one third of total CD4⁺ T cells at that time-point. Depletion of CD25⁺ cells at the peak of EAE inhibited the recovery, and depletion after the recovery removed the natural resistance to EAE re-induction. Moreover, the transfer of a small number of highly activated CNS-derived Treg, and not polyclonal Treg, partially suppressed EAE suggesting the importance of the activation status and antigen reactivity in Treg-mediated suppression (McGeachy et al., 2005). The accumulation of Treg within the CNS during EAE was later shown to be associated with rapid proliferation of Treg driven by the inflamed CNS itself (O'Connor et al., 2007) and not with *de novo* induction of Foxp3⁺ cells (Korn et al., 2007). The CNS-derived Treg were reported to be enriched for myelin-reactive Treg (O'Connor et al., 2007, Korn et al., 2007), and were found to suppress IFN- γ production by CNS-derived effector T cells *in vitro*, but not IL-17 secretion (O'Connor et al., 2007). High levels of IL-6 and TNF- α in the inflamed CNS were suggested to adversely affect Treg-mediated suppression (Korn et al., 2007).

1.7.2.4. Therapeutic transfer of Treg

In the light of the potential of Treg to inhibit EAE, further investigations have been undertaken to determine the exact mechanism of Treg-mediated suppression when given as a prophylactic measure but also to explore the therapeutic application in an on-going disease. Naïve and *in vitro* expanded MBP-reactive, but not polyclonal, nTreg were demonstrated to suppress MBP-induced EAE and moderately improve PLP-induced EAE when given prior to EAE induction (Stephens et al., 2009). Moreover, they were able to ameliorate disease in a chronic model of EAE when given after disease onset. Importantly, a stable expression of Foxp3 was key to efficient suppression and it was only maintained by CD62L^{high} Treg reflecting superior potential of this Treg subset in suppressing pathogenic T cells. Another study has demonstrated that nTreg can inhibit EAE mediated by different autoantigens if they are first activated by their cognate antigen (Yu et al., 2005).

As nTreg are present at a relatively low frequency in the periphery and obtaining sufficient numbers of antigen-reactive cells has proved difficult, attention has been drawn to *in vitro* induced Treg (iTreg) which provide an easy means of generating robust numbers of antigen-reactive cells. Zhang et al. have demonstrated that PLP-reactive iTreg expand *in vivo* upon antigen exposure and efficiently suppress PLP-driven EAE when transferred before disease induction (Zhang et al., 2010). They have also shown that iTreg can only suppress EAE driven by the same peptide as their cognate antigen and hence are incapable of performing bystander suppression. Opposing results were found by a different group who demonstrated that polyclonal iTreg can efficiently suppress MOG-driven EAE when given prior to disease induction and ameliorate its severity when given after priming but before the onset of clinical signs of disease (Selvaraj and Geiger, 2008). IL-10 secretion and ability to induce ‘infectious tolerance’ were among suggested mechanisms of iTreg-mediated suppression (Selvaraj and Geiger, 2008).

To-date experimental results provide conflicting evidence as to how important is antigen reactivity in both nTreg and iTreg-mediated suppression of EAE. The mechanisms involved are equally unclear, however an important emerging fact is

that Treg subsets seem to differ in their efficacy to suppress T cell responses and this may have important implications in their potential use in therapy.

1.8. Regulatory CD4⁺ T cells in allergy

Harmful immune responses directed against self-tissues occur not only in autoimmune diseases but also in allergic responses to otherwise innocuous antigens. For an allergic reaction to occur, the first encounter with a foreign antigen must lead to the production of IgE antibody that sensitizes immune cells such as mast cells, and then upon every subsequent exposure to the same antigen, an immune reaction follows. Normally associated with protective immunity to parasites, IgE responses to harmless environmental antigens lead to allergies such as allergic rhinitis, food allergies or asthma. In this thesis asthma, which is a chronic inflammatory disease of the airways, will be discussed in more detail.

1.8.1. Asthma

Asthma is a complex chronic inflammatory disease of the conducting airways that develops in response to harmless environmental antigens, called allergens. It is one of the most prevalent chronic conditions in Western countries affecting 1 in 7 children and 1 in 12 adults, and frequently occurs concurrently with other allergies (Holgate et al., 2009). Although the antigens causing asthma are usually easily identifiable, the underlying pathology seems to be multifactorial and is still unclear.

1.8.1.1. Symptoms and underlying causes of asthma

Allergic asthma is characterised by reversible obstruction of airways and airway hyperresponsiveness (AHR) leading to symptoms such as wheeze, cough, shortness of breath and chest tightness. The allergic reaction can be divided into two phases with the first one occurring within minutes of encountering an allergen and resulting in bronchospasm which includes mucosal oedema and airway narrowing (Wills-Karp, 1999). The second phase occurs within hours of exposure and is associated with inflammation, persistent airway obstruction and AHR.

In terms of asthma etiology, there is evidence of *in utero* influences and early life events that confer a risk of developing asthma later in life (Warner, 2004). Suboptimal foetal growth, maternal micronutrient deficiencies (such as retinoic acid) and type of diet, maternal smoking and maternal atopy are all among factors associated with an impaired infant lung function and later development of asthma (Cardoso et al., 1995, Warner, 2004). There is also a suggested inverse correlation between early exposure to microbes and a reduced risk of developing allergies including asthma (Holgate et al., 2009). Evidence implies that at birth, an infant's immune responses are skewed towards Th2 as these are protective in pregnancy (Warner, 2004) and an early encounter with endotoxins helps to even out the imbalance between Th1 and Th2 responses. This is the basis of the hygiene hypothesis suggesting that reduced exposure to microbes observed in industrialised countries is responsible for the skewed Th2 responses and hence increased incidence of allergies (Strachan, 1989, Holgate et al., 2009).

Asthma has been also shown to have strong genetic links. In contrast to MS, the HLA-associations are less evident in asthma but HLA-DR, HLA-DQ, HLA-DP and HLA-G were suggested by some studies to be among the susceptibility haplotypes (Nicolae et al., 2005, Shiina et al., 2004). The genes implicated in asthma pathogenesis can be broadly subdivided into four categories (Holloway et al., 2010) with the first one representing genes directly modulating the responses to environmental exposures such as TLRs which are expressed on innate immune cells and detect microbes (Yang et al., 2006). The second group comprises genes implicated in the maintenance of the epithelial integrity at the mucosal surfaces such as *flg* encoding filagrin (Rodriguez et al., 2009). The third group is mainly associated with the genes that regulate immune cell responses primarily Th1 and Th2 differentiation/function such as *gata3*, *stat6*, *il13*, *il4R* and *tbx21* (Holloway et al., 2010). The last group features the genes implicated in the responses of tissue to chronic inflammation such as airway remodelling and examples include a disintegrin and metalloproteinase (*adam3*) and *pde4d*.

1.8.1.2. Immunopathology of asthma

A combination of genetic and environmental factors is believed to be the reason why some people react to innocuous inhaled antigens whereas others remain tolerant to them. Several hundreds of allergens have been identified from diverse sources such as house dust mite (HDM) faeces, pollen and animal dander. Although some of them share structural and functional similarities, it is still unclear what constitutes an allergen (Traidl-Hoffmann et al., 2009). It has been reported that some allergens, for example Der p1 (HDM), have an enzymatic activity which enables them to disturb the Th1/Th2 balance (Schulz et al., 1998) and to disrupt epithelial tight junctions (Wan et al., 1999). It is believed that the route of exposure, dose and function of an allergen are critical to the sensitization and vary with each individual (Traidl-Hoffmann et al., 2009). The immune responses implicated in asthma pathology are summarised in Figure 1.5 at the end of this section.

1.8.1.2.1. CD4⁺ T cells orchestrate inflammatory reaction

Allergen sensitization occurs via immunoglobulin-E (IgE) production which has been closely linked to asthma severity (Burrows et al., 1989) and initial and sustained responses to allergen (Busse and Lemanske, 2001). For the IgE response to occur, an inhaled allergen is taken up by DC that line mucosal airway surfaces and presented to T cells and B cells in draining lymph nodes (Holt et al., 1990, Lambrecht and Hammad, 2009). The mechanism of DC activation is thought to involve an allergen itself as it was demonstrated that HDM allergens can activate TLR signalling and induce local release of cytokines that not only skew the immune response towards Th2 but also promote DC maturation and activation (Trompette et al., 2009, Hammad et al., 2009).

CD4⁺ T cells are strongly implicated in the pathogenesis of asthma as the increased numbers of T cells have been consistently observed in bronchoalveolar lavage (BAL) fluids and bronchial biopsies from asthmatic patients (Gerblich et al., 1991, Azzawi et al., 1990). Distinct CD4⁺ T activation status and cytokine profile has been reported in BALF and bronchial biopsies of asthmatic patients versus peripheral blood and

this includes increased expression of CD25 and Th2-associated cytokines such as IL-4, IL-5, IL-13 and GM-CSF (Walker et al., 1992, Robinson et al., 1992, Del Prete et al., 1993). A positive correlation has been made between the levels of these cytokines and the levels of IgE and eosinophils in the lung tissue which strongly suggests their pathogenic role in asthma (Robinson et al., 1993, Wills-Karp, 1999). Recently, a Th17 cell subset was also implicated in the most severe, often neutrophilic, forms of asthma as several studies reported IL-17 expression in airway biopsies and sputum of asthmatic patients (Bullens et al., 2006, Al-Ramli et al., 2009).

Th2 cells are thought to play a critical role in orchestrating immune responses in asthma as the cytokines they produce not only promote Th2 differentiation and IgE synthesis but also have been shown to contribute to activation of mast cells, and recruitment and activation of neutrophils and basophils (Wills-Karp, 1999). Mast cells, eosinophils and basophils are large secretory cells that upon activation release granules containing toxic and pro-inflammatory molecules such as histamine, cytokines and cationic proteins (O'Donnell et al., 1983, Welle, 1997). As mentioned in section 1.2.1, the main role of these cells is defence against extracellular parasites but in asthma they have been reported to enhance activation of other immune cells, cause microvascular leakage, increase mucus production, bronchoconstriction and AHR (Gundel et al., 1991, Metcalfe et al., 1997).

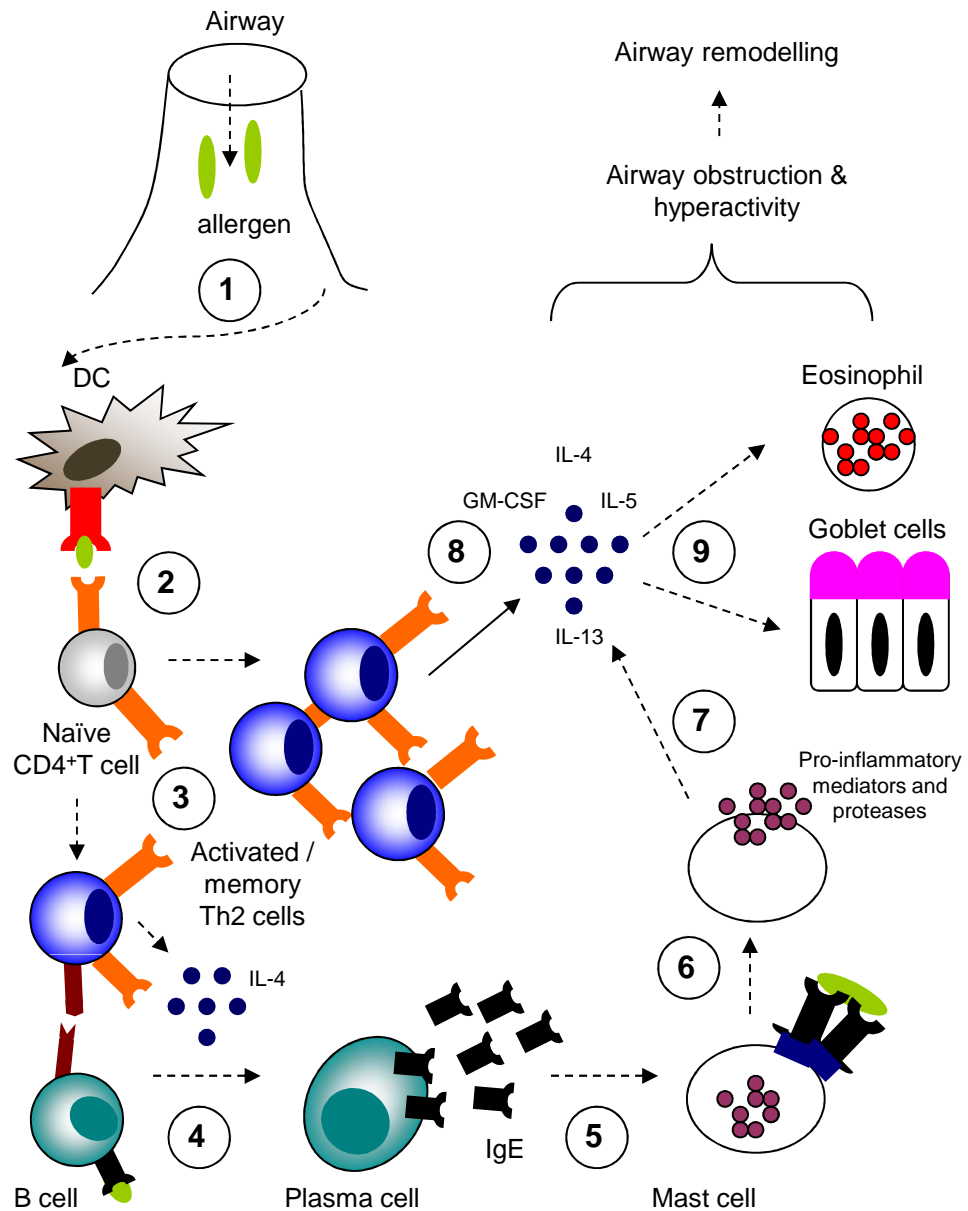


Figure 1.5 Immunopathogenesis of asthma.

Schematic diagram of immune responses thought to contribute to asthma pathology. (1) Upon first allergen exposure, DC lining up mucosal surfaces in the lung take up antigen and (2) migrate to draining lymph nodes to present it to T cells. (3) Activated CD4⁺ T cells differentiate into Th2 cells and produce (4) IL-4 which induces an antibody class switch to IgE. (5) IgE binds to and sensitizes mast cells. (6) Upon every subsequent allergen exposure, allergen binds to IgE and induces mast cell degranulation and release of (7) pro-inflammatory mediators such as proteases, growth factors and cytokines. (8) In addition, memory Th2 cells, generated during the first allergen exposure, become re-activated and produce pro-inflammatory cytokines such as IL-4, IL-5 and IL-13 leading to (9) goblet cell hyperplasia, recruitment of eosinophils from the bone marrow, damage to airway epithelium and microvascular leakage. This results in mucus production, airway obstruction and hyperactivity eventually leading to airway remodelling.

1.8.1.2.2. Breakdown in regulatory mechanisms

There is strong experimental evidence for the induction of Treg populations with low dose inhaled antigen in the absence of adjuvant (Akbari et al., 2002, Ostroukhova et al., 2004) which suggests an important role for *in vivo* allergen-induced Treg in natural protection against sensitization in addition to thymically-derived nTreg. Reduced numbers and impaired function of CD4⁺ CD25⁺ T cells as well as decreased Foxp3 expression have been reported specifically within the lung compared to the blood of asthmatic children (Hartl et al., 2007). A different study observed unaltered numbers of CD4⁺ CD25⁺ T cells but decreased Foxp3 expression in the peripheral blood of adult asthmatic patients, however Treg function was not assessed (Provoost et al., 2009).

An important mechanism of Treg-mediated suppression is the secretion of anti-inflammatory IL-10, and levels of IL-10 production appear to be relevant to asthma pathology (O'Garra et al., 2008). Several studies have reported decreased IL-10 levels in BAL fluid (John et al., 1998) and reduced numbers of IL-10⁺ T cells in the peripheral blood of asthmatic patients versus healthy controls (Akdis et al., 2004).

1.8.1.3. Current treatments

Most current treatments of asthma rely on the long-term suppression of airway inflammation by corticosteroids or on the use of bronchodilators (van der Velden, 1998). Corticosteroids are potent inhibitors of inflammation and they were shown to function by directly suppressing expression of genes encoding pro-inflammatory mediators such as IL-1 β or GM-CSF and promoting expression of genes coding for anti-inflammatory mediators such as IL-10 (Mozo et al., 2004). Although the treatment with corticosteroids is effective and has been shown to suppress bronchial inflammation and improve lung function (Booth et al., 1995), these drugs act in an antigen non-specific fashion, leaving the patient significantly immunocompromised and prone to opportunistic infections. Another type of treatment used in conjunction with corticosteroids includes short-acting β 2-agonists such as salmeterol. β 2-agonists act on β 2-adrenergic receptor and induce smooth muscle relaxation resulting in

bronchodilatation (Woolcock et al., 1996). Leukotriene-receptor antagonists are also used and have been shown to control bronchoconstriction and airway inflammation (Fanta, 2009).

Newer approaches in asthma therapy target IL-4, IL-5 and IgE. Soluble recombinant IL-4 receptor delivered in a nebulized form has been demonstrated by several studies to be a safe and effective treatment of moderate asthma without the use of corticosteroids (Borish et al., 2001). IL-5 blocking monoclonal antibody was shown to reduce the number of eosinophils in patients' sputum but did not affect AHR (Leckie et al., 2000). Another blocking compound is a humanized monoclonal antibody (omalizumab) that forms complexes with free IgE and hence blocks IgE from binding to mast cells and basophils (Busse and Lemanske, 2001). It has been shown to effectively attenuate allergen-induced airway obstruction and eosinophil accumulation in the airways and has been approved for use in adults and children (Fahy et al., 1997, Fanta, 2009).

The fact that most of current asthma treatments target the immune system emphasizes the important role of inflammation in the disease pathogenesis. However antigen non-specific immunosuppression has proven to have many side effects and has a limited efficacy. That is why a large amount of work is currently going into investigation of allergen-specific immunotherapies and animal models of allergic airways inflammation (AAI) provide a very useful tool to verify their potential.

1.8.2. Allergic airways inflammation as a model of asthma

Allergic airways inflammation is an animal model of allergic asthma and can be induced in a variety of species such as guinea pigs, rats and mice. Similarly to human asthma, AAI is characterized by infiltration of the lung with mast cells, lymphocytes and eosinophils, airway hyper-responsiveness and mucus production. The most widely used species are mice and as they do not develop asthma spontaneously, the disease is induced in them artificially. The allergens include the well-known human allergens such as HDM or animal dander but also chicken ovalbumin (OVA). There

are many different murine models of allergic asthma and each of them represents certain morphological and functional features of the human disease (Nials and Uddin, 2008).

1.8.2.1. Immunopathology

AAI can be induced in mice by sensitization with an antigen in adjuvant consisting of aluminium hydroxide and magnesium hydroxide (alum) in order to induce IgE synthesis and Th2 memory response and subsequent airway challenges with the antigen (Leech et al., 2007). The disease can also be transferred by activated TCR transgenic antigen-specific T cells polarised towards Th2 phenotype *in vitro* or by CD4⁺ T cells isolated from antigen-sensitized animals into naïve recipients and subsequent antigen airway challenges (Cohn et al., 1997, Hogan et al., 1998) supporting the hypothesis that CD4⁺ T cells drive and orchestrate the inflammation in AAI.

The direct evidence of the causal role for CD4⁺ T cells in driving the inflammation in AAI came from the cell depletion studies which showed that depleting CD4⁺ T cells with a monoclonal antibody prior to airway challenges with the antigen suppressed lung eosinophilia and AHR (Gavett et al., 1994), and attenuated subepithelial fibrosis and mucus cell metaplasia (Foster et al., 2002). Conversely, the depletion of CD8⁺ T cells did not have an effect on AAI (Gonzalo et al., 1996). More recently, it was demonstrated that depletion of CD4⁺ T cells during chronic AAI results in a reduced airway inflammation but does not affect progressive airway remodelling (Doherty et al., 2009). This observation highlights the differences among variable AAI models and suggests that CD4⁺ T cells could have different roles at different stages of the disease.

CD4⁺ T cells driving AAI are thought to be Th2 cells. Abolishing the synthesis of Th2 signature cytokines, IL-4 and IL-5, either via monoclonal antibody approach or genetic manipulation, has shown that these cytokines play a major role in disease pathogenesis (Wills-Karp, 1999). IL-5 was shown to be important for differentiation,

migration and activation of eosinophils (Weller, 1991, Ohnishi et al., 1993) and its blockade prior to antigen airway challenge in OVA-induced model of AAI resulted in a reduced eosinophil mobilization and hence decreased numbers of eosinophils in the lung and BALF (Hogan et al., 1997). Targeting IL-4 during sensitization in AAI revealed that it is critical for IgE and IL-5 synthesis, and for the development of lung eosinophilia (Coyle et al., 1995). The importance of IL-4 in AAI is further highlighted by the discoveries that it regulates Th2 cell differentiation (Zheng and Flavell, 1997) and induces antibody class switch to IgE during B cell activation (Kashiwada et al., 2010). Another Th2 signature cytokine, IL-13, was shown to be implicated in goblet cell development, mucus cell metaplasia and AHR (Yang et al., 2001). Finally, targeting the master regulator of Th2 lineage, GATA-3, was found to significantly inhibit the development of AAI (Zhang et al., 1999).

What causes an aberrant Th2 cell activation and expansion is still unclear. As discussed in section 1.2.3.5 the cytokines produced by APC during T cell activation dictate the T cell differentiation pathway. There are several types of APC present in the airways and these include alveolar macrophages and DC (Wills-Karp, 1999). A subset of intraepithelial DC lining the mucosal surfaces in the lung has been shown to efficiently take up an inhaled allergen, migrate to the draining mediastinal lymph nodes, present it to T cells and induce Th2 phenotype (Hammad et al., 2010). Interestingly, previously implicated in this process basophils (Sokol et al., 2009) were shown to be important in amplifying T cell responses but redundant in initiating them (Hammad et al., 2010). DC implicated in the induction of Th2 responses in AAI were termed ‘inflammatory’ DC and their influx into the airways during inflammation has been reported, however the driving/activating signal is unclear (Lambrecht and Hammad, 2009). One of the cytokines thought to be involved in DC activation in AAI is IL-33 (Besnard et al., 2011).

Primary antigen exposure leads to the generation of Th2 cells and synthesis of IgE which binds to high affinity FcεR1 receptors on the surface of mast cells in the lung (Wills-Karp, 1999). Upon every subsequent antigen exposure, antigen binds to pre-bound IgE on mast cells leading to FcεR1 receptors cross-linking and mast cell

degranulation. The mediators released by mast cells include histamine, prostaglandins, serine proteases, chemokines and cytokines such as TNF- α and IL-5 which contribute to the recruitment of eosinophils from the bone marrow, increased vascular permeability and mucus production (Stone et al., 2010). Th2 cells have been shown to be re-stimulated in the airways by DC and basophils (Lambrecht and Hammad, 2009) to produce Th2-associated cytokines and chemokines promoting an influx and activation of eosinophils and other immune cells. Activated eosinophils produce a range of cytotoxic molecules, cytokines and growth factors which damage airway epithelium and can induce AHR and influence airway remodelling in chronic models of AAI (Hogan et al., 1997, Bochner and Gleich, 2010).

Recently, a Th2-independent mechanism of asthma pathology has been discovered. In addition to Th2 cells being an important source of IL-13, a novel type of innate lymphoid cell identified by the McKenzie group was found to produce high levels of IL-13 as well (Neill et al., 2010). Due to their ability to produce IL-13 and 'nu' being the 13th letter of the Greek alphabet, these innate cells were termed nuocytes and were shown to be critical in mediating type-2 responses responsible for worm expulsion in helminth infection (Neill et al., 2010). Soon after, nuocytes have been demonstrated to contribute to AHR in a murine model of virus-induced asthma (Chang et al., 2011) and in a murine model of OVA-induced AAI (Barlow et al., 2012).

1.8.2.2. Models of AAI

As mentioned already there is a wide variety of antigens used as allergens to induce AAI and the most common include antigens derived from HDM extracts and OVA. The model of OVA-induced AAI is particularly useful as TCR transgenic mice for OVA-derived peptide OVA₃₂₃₋₃₃₉ are available which enable tracking and investigating allergen-specific T cells. Those are OT-II mice (haplotype H-2^b) (Barnden et al., 1998) on C57BL/6 background or DO11.10 mice (haplotype H-2^d) (Murphy et al., 1990) on BALB/c background.

AAI is most commonly induced by intraperitoneal injection of an antigen in alum which marks the sensitization phase and subsequent airway challenge with the antigen which is given either intranasally or intratracheally (Nials and Uddin, 2008). Alum was originally thought to act as an antigen depot enabling the antigen to be slowly released over long periods of time (Marrack et al., 2009). However, it is now known that alum exerts variety of immunomodulatory functions, for example it activates and recruits innate immune cells such as DC to the site of injection (Marrack et al., 2009). AAI can also be induced by an adoptive transfer of activated TCR transgenic antigen-specific T cells polarised towards Th2 phenotype *in vitro* or by CD4⁺ T cells isolated from antigen-sensitized animals into naïve recipients followed by antigen airway challenges (Cohn et al., 1997, Hogan et al., 1998).

Mouse models of AAI can also be divided into acute and chronic, each representing certain morphological and functional features of the human disease (Nials and Uddin, 2008). Acute AAI models are usually self-resolving and are useful in investigating the pathogenesis of the inflammatory responses in asthma. They reproduce many key features of allergic asthma such as elevated levels of IgE, airway inflammation, mucus cell metaplasia and AHR. However, in order to investigate the chronic features of asthma such as persistent AHR and airway remodelling, a number of chronic models of AAI were established and these are mostly based on long-term antigen exposure (Wegmann, 2008).

1.8.2.3. Regulation of AAI by Treg

One of the first studies implicating regulatory T cells in the control of Th2-driven lung inflammation in AAI showed that low-dose antigen exposure in the absence of adjuvant induces high IL-10 expression by previously transferred OT-II CD4⁺ cells into naïve recipients (Akbari et al., 2002). Adoptive transfer of these ‘tolerogenic’ IL-10⁺ T cells into OVA-sensitized mice significantly inhibited antigen-induced IgE levels, AHR and eosinophilia in the BALF. This was followed by the report from Jaffar et al. who demonstrated that depleting Th2-polarised OT-II cells of CD4⁺

CD25⁺ T cell population prior to transfer into naïve hosts and airway challenges with OVA significantly exacerbated the disease (Jaffar et al., 2004).

The direct evidence that Treg control AAI was given by Kearley et al. who showed that the transfer of OVA-reactive CD4⁺ CD25⁺ cells prior to antigen airway challenge reduced AHR, lung eosinophilia and decreased expression of Th2-associated IL-5 and IL-13 in the lung (Kearley et al., 2005). Moreover, this protective effect was reversed by blockade of IL-10, however IL-10-deficient Treg were still capable of improving AAI by enhancing IL-10 production from recipient CD4⁺ T cells (Kearley et al., 2005). This suggested that Treg could exert their suppressive function via 'infectious tolerance' mechanisms in the context of not only Th1- but also Th2-mediated inflammation. A different study used a cell depletion approach and showed that anti-CD25-mediated Treg depletion results in exacerbated AAI and implied that Treg mediate their protective effects via suppression of DC activation (Lewkowich et al., 2005).

A model of acute self-resolving AAI demonstrated that Foxp3⁺ Treg accumulate in the lung and draining mediastinal lymph nodes upon antigen exposure and are critical to the resolution of inflammation (Leech et al., 2007). The depletion of Treg using anti-CD25 before antigen airway challenge led to the increased lung eosinophilia and enhanced allergic responses. On the other hand, an adoptive transfer of polyclonal CD4⁺ CD25⁺ Treg suppressed IgE levels, eosinophilia and levels of Th2-associated cytokines in the lung in an IL-10 independent manner (Leech et al., 2007).

Newer approaches of assessing Treg function involve the use of DERE mice which express eGFP and diphtheria toxin receptor (DTR) under the *foxp3* promoter (Lahl et al., 2007), and administration of diphtheria toxin (DTx) in this system enables selective depletion of Foxp3⁺ cells. Baru et al. have confirmed the significance of Treg function in early phases of AAI by selectively depleting Foxp3⁺ Treg during the sensitization phase and demonstrated that this leads to significantly exacerbated disease (Baru et al., 2010).

1.8.2.4. Therapeutic transfer of Treg

As Treg have been shown to be capable of suppressing AAI, further work has been undertaken to determine the potential of *in vitro* expanded nTreg and *in vitro* generated iTreg in both prophylactic and therapeutic application. The transfer of TGF- β -converted polyclonal CD4⁺ CD25⁻ T cells prior to sensitization and airway challenge with HDM antigen was shown to decrease inflammatory cell infiltration in the lung and reduce the levels of Th2 cytokines produced by splenocytes upon re-stimulation *in vitro* (Chen et al., 2003). These promising results are further supported by the recent findings from Xu et al. who demonstrated that both *in vitro* expanded polyclonal nTreg and TGF- β -induced polyclonal iTreg can improve AAI when transferred prior to antigen challenge (Xu et al., 2012). In that study increased percentage of total Foxp3⁺ cells and decreased percentage of DC in mediastinal lymph nodes were observed suggesting a possible mechanism of suppression. Moreover, polyclonal nTreg and iTreg transfer after the first antigen airway challenge decreased AAI severity indicating that Treg could be effective at the very early stages of the disease (Xu et al., 2012).

Efficacy of Treg-mediated suppression in already developed AAI was shown in mice with an already established AHR that were treated with polyclonal CD25⁺ and CD25⁻ T cells isolated from the spleen and lung of naïve mice (McGee and Agrawal, 2009). Both Treg subsets from the spleen and lung were shown to reverse AHR, decrease lung infiltration, mucus production and eosinophilia in the BALF, reduce the levels of Th2 cytokines and increase IFN- γ in the BALF. However, an inability to track Treg in that study did not allow for more detailed analysis of their function and phenotype.

To date, there is no experimental evidence on the importance of the antigen reactivity in both nTreg- and iTreg-mediated suppression of lung inflammation in AAI which may have important implications in their potential use in therapy. Similarly, the stability of Treg suppressive phenotype in Th2-mediated inflammation *in vivo* and the inhibitory mechanisms involved are unclear, however, they seem to vary in different AAI models.

1.9. Treg in clinical trials

Human nTreg similarly to their murine counterparts express CD4, CD25 and Foxp3. However, as activated T cells were shown to transiently upregulate Foxp3 expression, other markers are used to distinguish them from activated conventional T cells such as low/absent expression of IL-7 receptor (CD127) (Liu et al., 2006, Seddiki et al., 2006). Isolation of live highly pure human regulatory T cells from the peripheral blood is still very problematic and there is a continuous search for better more specific cell surface markers that would reduce the risk of transferring either unstable or contaminated Treg populations (Edinger and Hoffmann, 2011). Protocols for efficient *in vitro* expansion of human nTreg have been established (Hoffmann et al., 2004, Hippen et al., 2011), however variable loss of Foxp3 expression is being reported (Hoffmann et al., 2009, Putnam et al., 2009). The use of pharmacological agents such as rapamycin which is thought to selectively promote expansion of Foxp3⁺ cells has been suggested to improve the yield of more pure Treg populations (Strauss et al., 2007). Additionally, sorting for CD45RA⁺ Treg prior to expansion has been shown to generate more stable Foxp3⁺ Treg population (Miyara et al., 2009).

Most clinical trials using Treg have been conducted in graft versus host disease (GvHD). In one of the first small phase I clinical trials, the stem cell transplantation (SCT) group from the University of Perugia, Italy, used freshly isolated donor CD4⁺ CD25⁺ Treg and infused them into 28 patients receiving a haploidentical graft without an additional immunosuppressive treatment (Di Ianni et al., 2011). The study found that 26 out of 28 patients remained free of clinically relevant GvHD at the median follow-up time of 11.2 months and 2 other patients developed grade II acute GvHD. A different phase 1 clinical trial led by Blazar and colleagues involved *in vitro* expanded CD4⁺ CD25⁺ T cells isolated from partially HLA-matched third-party umbilical cord blood (Brunstein et al., 2011). Transfused Treg were detectable in all 23 GvHD patients, who co-received immunosuppression at that time, two weeks after the cell transfer and a reduced incidence of grade II to grade IV acute GvHD was observed.

In a more relevant setting to autoimmune disease, the results of the phase I/II clinical trial with antigen-specific Treg in Crohn's disease have been recently presented (MedicalNewsToday, 2011). In that open-label and uncontrolled trial the drug OvaSave® by TxCell SA was given as a single dose to 20 patients with severe Crohn's disease. 38% of patients were reported to go into remission 5 weeks after the treatment. The drug has been now moved into a controlled phase II study. Another, much larger phase 1 clinical trial in type 1 diabetes (T1D) led by Bluestone and colleagues at the University of California is currently recruiting patients to investigate the therapeutic potential of *in vitro* expanded autologous CD4⁺ CD25⁺ CD127^{low/-} polyclonal Treg (ClinicalTrials.gov, 2012).

The initial results from phase I clinical trials indicate that Treg therapy has a potential to be safe and effective, however thorough investigation into Treg phenotype, function and stability in different disease settings is required. The question of paramount importance is whether Treg have to be antigen-reactive to exert their immunomodulatory functions in the most efficacious way. Moreover, optimisation of the techniques that will enable pure, efficient and cost-effective generation of Treg is critical to the advancement of cellular therapies (Edinger and Hoffmann, 2011, Wang et al., 2011).

1.10. Specialized subsets of Treg - do Treg adjust to the type of inflammation?

It is well established that conventional CD4⁺ T cells upon stimulation and cytokine signalling received from the DC follow different differentiation pathways which are optimized to fight against different types of pathogens. It is also known that CD4⁺ T cells responses and the inflammation they orchestrate are controlled by regulatory T cells to make sure there is no excessive damage to the host tissues. The question which follows these two important features of the immune response and may have important implications in immunotherapy is whether Treg themselves can specialize to better control different types of inflammation.

Treg can express tissue-specific homing receptors and chemokine receptors which control their trafficking and localization to different organs, and this differential migration capacity was suggested to be critical for their *in vivo* suppressive functions (D'Ambrosio et al., 2001, Campbell and Koch, 2011). Thymically-derived Treg display a naïve-like phenotype, lacking expression of adhesion molecules or chemokine receptors, whereas activation of Treg in skin-draining lymph nodes (LN) and gut allows acquisition of a 'memory-like' phenotype (Siewert et al., 2007). These activated Treg can be distinguished through the expression of $\alpha 4\beta 7$ integrin (gut-associated mesenteric LN) versus P- and E-selectin ligands (skin-draining LN) that control migration to the appropriate tissue *in vivo*. To date, multiple studies have reported on the differential expression of receptors and ligands by Treg depending on the environment they were activated in (Kohm et al., 2002, Piccio et al., 2002, Muller et al., 2007).

More recently, transcription factors previously thought to be expressed specifically in effector $CD4^+$ T cells were identified in Treg as well. The expression of T-bet, which is the master regulator driving the Th1 phenotype, was detected in Treg in a model of Th1-mediated infection, and was found to be critical for Treg migration to the site of infection, homeostasis and function (Koch et al., 2009). Similarly, Treg lacking interferon regulatory factor-4 (IRF-4), a transcription factor essential for Th2 effector differentiation (Lohoff et al., 2002), were reported to fail to control Th2 responses and lead to autoimmune lymphoproliferative disease (Zheng et al., 2009). Both of these studies suggested that Treg utilize components of the transcriptional machinery responsible for driving particular type of the $CD4^+$ T cell lineage and use them in their favour to regulate the corresponding type of $CD4^+$ T cells during inflammatory response.

1.11. Hypothesis and aims

The central hypothesis of this project is that Treg acquire a different phenotype in Th1- versus Th2-mediated inflammatory diseases in-line with their effector CD4⁺ T cell (Teff) counterparts and can therefore optimally specialize to control such Teff responses.

The aims of this project were to:

- 1) Determine whether Treg specialization occurs *in vivo* by comparing CNS-derived Treg in Th1/Th17-mediated EAE versus lung-derived Treg in a model of Th2-mediated AAI.
- 2) Test if Treg can be polarised *in vitro* and hence recapitulate *in vivo* observations, and establish what conditions are most optimal.
- 3) Assess whether antigen responsiveness or specialized phenotype are the key factors in the efficacy of Treg function in EAE and AAI.
- 4) Establish and characterize a model of MOG-induced AAI which will allow testing aim 3.
- 5) Investigate the mechanism of Treg-mediated suppression in EAE and AAI.

2 Materials and Methods

2.1. Mice

C57BL/6 (H-2^b background) mice were either purchased from Harlan Laboratories Ltd (Shardlow, UK) or were bred under specific pathogen-free conditions at the University of Edinburgh. 2D2 (CD90.1, CD90.2 or CD90.1/CD90.2) (Bettelli et al., 2003), MOG^{-/-} (CD45.1) (Delarasse et al., 2003), Foxp3-GFP (CD90.2) (Fontenot et al., 2005), OT-II (CD45.1) (Barnden et al., 1998) and Foxp3.LuciDTR-4 (CD90.1, CD90.2) (Suffner et al., 2010) mice (all on H-2^b background) were bred under specific pathogen-free conditions at the University of Edinburgh. 2D2 mice are transgenic for a TCR reactive to MOG₃₅₋₅₅. OT-II mice are transgenic for a TCR reactive to OVA₃₂₃₋₃₃₉. Foxp3.LuciDTR-4 mice express eGFP, diphtheria toxin receptor (DTR) and luciferase under the *foxp3* promoter. 2D2xFoxp3.LuciDTR4 mice were generated at the University of Edinburgh by crossing 2D2 mice with Foxp3.LuciDTR4 mice. IL-4-GFP reporter mice were kindly provided by Dr M. Taylor. All mice used were female aged 6-12 weeks of age, unless otherwise specified. All experiments had University of Edinburgh ethical approval and were performed under UK legislation.

2.2. Peptides and proteins

Myelin oligodendrocyte glycoprotein peptide 35-55 of MOG protein (MOG₃₅₋₅₅; MEVGWYRSPFSRVVHLYRNNNGK) was synthesised by Cambridge Research Biochemical (Cambridge, UK). Recombinant MOG (rMOG) was synthesised using an *Escherichia coli* expression system [ORIGAMI (DE3)] (Fillatreau et al., 2002) and was tested for the presence of LPS with E-toxate kit (Sigma-Aldrich, Poole, UK). Chromatographically purified chicken ovalbumin (OVA) was obtained from Worthington Biochemical Corporation (Lakewood, USA). OVA peptide 323-339 of OVA protein (OVA₃₂₃₋₃₃₉; ISQAVHAAHAEINEAGR) was synthesized by PepLogic (Essex, UK).

2.3. Adjuvants

Complete Freund's adjuvant (CFA) containing 1 mg/ml heat-killed Mycobacterium Tuberculosis H37Ra (MTB) was purchased from Sigma-Aldrich. Imject Alum containing 40mg/ml of aluminium hydroxide and 40mg/ml magnesium hydroxide was obtained from Pierce (Rockford, USA).

2.4. General reagents

2.4.1. Wash buffer

RPMI 1640 medium containing 25mM Hepes (Gibco, Paisley, UK).

2.4.2. RPMI-5% and RPMI-10%

RPMI 1640 medium, supplemented with 2mM Glutamine (PAA Laboratories Ltd, Somerset, UK), 100U/ml Penicillin (PAA), 100µg/ml Streptomycin (PAA), 50µM 2-mercaptoethanol (Gibco) and 5% or 10% heat-inactivated fetal calf serum (FCS, Labtech, East Sussex, UK).

2.4.3. MACS buffer

HANKS balanced salt solution (PAA) supplemented with 2% heat-inactivated FCS (Labtech, Ringmer, UK).

2.4.4. FACS buffer

Phosphate-buffered saline solution (PBS) supplemented with 2% heat-inactivated FCS (Labtech) and 0.1% sodium azide (Sigma).

2.4.5. X-VIVO serum-free medium

X-VIVO 15 serum-free medium (BioWhittaker, USA) supplemented with 2mM Glutamine (PAA) and 50µM 2-mercaptoethanol (Gibco).

2.5. Cell isolation, purification and sorting

2.5.1. Isolation of cells from spleen and lymph nodes

Single cell suspensions from spleen and lymph nodes (LN) were obtained by mashing through sterile gauze and re-suspending in wash buffer. Cells were then centrifuged at 350×g for 5 min and resuspended in fresh wash buffer. All subsequent wash steps followed the same protocol. Splenic red blood cells (RBCs) were lysed at room temperature (RT) with RBC lysis buffer (Sigma) and washed once. Cells were counted using trypan blue (Sigma) exclusion.

2.5.2. Isolation of cells from CNS

Mice were sacrificed with CO₂ asphyxiation and perfused through the eyes with 10ml cold PBS. The brain was removed by dissection, and the spinal cord by intrathecal hydrostatic pressure. Tissues were cut into small pieces and further disrupted using a 1ml syringe prior to digestion with 2.5mg/ml collagenase (Lorne Laboratories, Reading, UK) and 1mg/ml deoxyribonuclease (Sigma) at 3:1 ratio for 40 minutes at 37°C. Mononuclear cells were isolated from the interface of a 30:70% discontinuous Percoll gradient (GE Healthcare, Stockholm, Sweden) by centrifugation at 850×g for 20 minutes without brake, and washed in RPMI. All wash steps were done at 300×g for 5 minutes. RBCs were lysed if needed in RBC lysis buffer. Cells were counted using trypan blue (Sigma) exclusion.

2.5.3. Isolation of cells from lung

Mice were sacrificed by intraperitoneal injection of pentobarbitone and perfused through the right ventricle with 15ml of cold PBS. In most experiments following perfusion and prior to fixation, the left lobe was tied off, removed and used for cell isolation. Lung tissue was finely chopped and digested in 0.23mg/ml collagenase (Type I-AS, Sigma-Aldrich) for 60 minutes at 37°C. Tissue was then disaggregated using rapid flushing through a 20G needle. Cell suspensions were washed twice in PBS, lysed in RBC lysis buffer (Sigma-Aldrich) and passed through a 40µm cell strain strainer prior to counting using trypan blue (Sigma-Aldrich) exclusion. All wash steps were done at 300×g for 5 minutes.

2.5.4. Purification of CD4⁺ T cells

Cells were isolated as described in 2.5.1 and incubated in 45µl of MACS buffer and 5µl of anti-CD4 conjugated magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) per 10^7 cells for 15 minutes at 4°C. Cells were washed in MACS buffer and resuspended at 2×10^8 cells/ml of MACS buffer. CD4⁺ cells were positively selected using an autoMACS Pro Separator or LS column and MidiMACS separator as per manufacturer's instructions (Miltenyi Biotec). Purity of CD4⁺ T cells was routinely $95\% \pm 5\%$.

2.5.5. Purification of CD4⁺ CD62L⁺ naïve cells by AutoMACS Pro Separator

Cells were isolated as described in 2.5.1 and purified using CD4⁺ CD62L⁺ T cells isolation Kit II (Miltenyi Biotec) according to manufacturer's instructions. Briefly, cells were incubated in 400µl of MACS buffer with 100µl of CD4⁺ T cells Biotin-Antibody Cocktail II for 10 minutes at 4°C. All volumes are given per 10^8 cells. Without washing, a further 300µl of MACS buffer and 200µl of Anti-Biotin MicroBeads were added for 15 minutes. Cells were then washed in MACS buffer, resuspended at 2×10^8 cells/ml of MACS buffer and negatively selected using AutoMACS (Miltenyi Biotec). Following washing, cells were resuspended in 800µl of MACS buffer and 200µl of CD62L MicroBeads and incubated for 15 minutes at 4°C. Cells were washed and positively selected using AutoMACS. Purity of CD4⁺ CD62L⁺ cells was routinely $90\% \pm 5\%$.

2.5.6. Sorting of CD4⁺ T cell subsets by FACS

CD4⁺ cells were purified as described in 2.5.4 and sorted by fluorescence-activated cell sorting (FACS) according to their GFP and/or cell surface marker expression. Post-sort purity was routinely $95\% \pm 5\%$. Cells were sorted using a Becton Dickinson (Franklin Lakes, USA) FACS ARIA II flow cytometer.

2.5.6.1. Sorting of nTreg from naïve Foxp3-GFP mice

Cells were stained with anti-CD4 and/or anti-CD62L (Table 2.1) and selected for CD4⁺ CD62L⁺ GFP⁺ cells.

2.5.6.2. Sorting of naïve CD4⁺ CD62L⁺ GFP⁻ (Vβ11⁺ or Vα3.2⁺) cells

If Foxp3.LuciDTR4 or Foxp3-GFP mice were used, cells were stained with anti-CD4 and anti-CD62L and selected for CD4⁺ CD62L⁺ GFP⁻ cells. When 2D2xFoxp3.LuciDTR4 mice were used, cells were stained with anti-CD4, anti-CD62L, anti-Vβ11 or anti-Vα3.2 and selected for CD4⁺ CD62L⁺ Vβ11⁺ (Vα3.2⁺) GFP⁻ cells. When non-Foxp3-GFP reporter mouse lines were used, cells were stained as above but not selected for GFP expression. The staining for Vβ11⁺ or Vα3.2⁺ cells was used to increase the purity of MOG-reactive cells within 2D2 cells.

2.5.6.3. Sorting of nTreg from the CNS/spleen of EAE mice and from naïve spleen for transfer

Cells were stained with anti-CD4 and anti-CD11b and selected for CD4⁺ CD11b⁺ GFP⁺ cells. Cells from the CNS were not pre-sorted for CD4⁺ cells as described in section 2.5.4.

2.6. *In vitro* cell culture and T cell polarisations

All cells were cultured at 37°C in a humidified atmosphere at 5% CO₂.

2.6.1. Th1, Th2, Th17 and GM-CSF-polarising conditions

Cells were obtained from spleen and/or peripheral LN as described in 2.5.1 and resuspended at 4x10⁶ cells/ml in RPMI-5%. Cells were cultured in 48-well plates when used for *in vitro* experiments, or 6-well plates (Corning Costar, High Wycombe, UK) when used for *in vivo* transfers. Cells were stimulated with 1µg/ml anti-CD3 (eBioscience, Hatfield, UK) or 10µg/ml MOG₃₅₋₅₅, unless otherwise stated. For Th1 polarisation, cells were cultured with 10U/ml rIL-2 (purified from the x63-IL-2 hybridoma, a gift from David Gray, University of Edinburgh), 25ng/ml rIL-12

and 25ng/ml rIL-18 (R&D Systems, Minneapolis, USA) for 72 hours. For Th2 polarisation, cells were cultured with 40U/ml rIL-2, 4ng/ml rIL-4 (Peprotech EC Ltd, London, UK), 5µg/ml anti-IL-12 (clone C17.8, BioXcell, New Hampshire, USA) and 5µg/ml anti-IFN-γ (clone XMG1.2, BioXcell) for 72 hours or 96 hours. For both polarisation protocols, cells were split after 48 hours and fresh polarising media (without the addition of anti-CD3 or MOG₃₅₋₅₅) was replenished. For Th17 polarisation, cells were cultured with 20ng/ml rIL-6, 20ng/ml rIL-23 and 3ng/ml rTGF-β (all R&D Systems) for 72 hours. For GM-CSF-skewing conditions, cells were stimulated with 5µg/ml anti-CD3 and 5µg/ml anti-CD28 (eBioscience) or 40µg/ml MOG₃₅₋₅₅ in the presence of 10µg/ml anti-IL-12 and 10µg/ml anti-IFN-γ for 72 hours (Codarri et al., 2011). Cells were split after 48 hours and fresh polarising media was replenished.

When sorted CD4⁺ T cells and nTreg were polarised, cells were resuspended at 4×10^5 cells/ml in RPMI-5% and stimulated with 2µg/ml of plate-bound anti-CD3 and anti-CD28 (eBioscience). Polarising conditions used were the same as described above, unless otherwise specified.

2.6.2. Generation of iTreg

Naïve CD4⁺ T cells were sorted using AutoMACS (see section 2.5.5) or FACS (see section 2.5.6.2) and resuspended in RPMI-10% at 3.75×10^5 cells/ml. Tissue culture 24-well plates (Corning Costar) were coated with 2µg/ml anti-CD3 and 2µg/ml anti-CD28 in PBS and incubated for 2 hours at 37°C. The plates were washed three times in PBS and seeded with 3.75×10^5 cells per well in the presence of 5ng/ml TGF-β and 100U/ml rIL-2. Cells were cultured for 120 hours. If the Foxp3.LuciDTR-4 or 2D2x Foxp3.LuciDTR-4 mouse lines were used, cells after 5-day culture were sorted for GFP⁺ cells using FACS.

2.6.3. Expansion of nTreg

nTreg were obtained as described in section 2.5.6.1 and resuspended at 1×10^6 cells/ml in RPMI-10%. Following resuspension, 1×10^5 cells were added per well to a

96-well round bottomed micro-titre plate (Corning Costar) at a 1:4 ratio with anti-CD3/anti-CD28 T cell expander Dynabeads (DynaL Biotech, Invitrogen, Paisley, UK) in the presence of 1000U/ml rIL-2. Cells were maintained at a concentration of $0.5 - 1 \times 10^6$ cells/well and cultured for 7 to 10 days.

2.6.4. Ex vivo re-stimulation assays

Cells were isolated as described in section 2.5 and resuspended in RPMI-5%. Splenocytes were resuspended at 8×10^6 cells/ml, dLN and cells from lung at 6×10^6 cells/ml and cells from CNS at $3 - 4 \times 10^6$ cells/ml, and cultured overnight with or without 20µg/ml MOG₃₅₋₅₅ and/or OVA₃₂₃₋₃₃₉ in 48-well or 96-well flat bottomed plates (Corning Costar). Brefeldin A (eBioscience) was added at 1:1000 dilution for the final 4 to 5 hours of culture, followed by intracellular cytokine staining.

2.6.5. Ex vivo recall cytokine assays

Cells were obtained from the spleen and dLN as described in section 2.5.1 and resuspended at 8×10^6 cells/ml or 6×10^6 cells/ml, respectively in X-VIVO medium. Cells were cultured in duplicate in 96-well flat bottomed micro-titre plates (Corning Costar) in the presence of increasing MOG₃₅₋₅₅ concentrations (0-30µM) for 72 hours. Cytokine levels in supernatants were measured by enzyme-linked immunosorbent assay (ELISA).

2.6.6. In vitro suppression assays

Sorted nTreg from naïve mice (2.5.6.1), *in vitro* expanded nTreg (2.6.3) or iTreg (2.6.2) were resuspended at 4×10^5 cells/ml in RPMI-10% and plated with either purified naïve CD4⁺ T cells or purified *in vitro* polarised CD4⁺ T effector cells (Tresp) at different ratios in triplicates in 96-well round bottomed micro-titre plates (Corning Costar). Irradiated splenocytes from C57BL/6 mice (resuspended at 4×10^6 cells/ml) were added to each well as antigen presenting cells. Cells were pulsed with 10ug/ml MOG₃₅₋₅₅ and cultured for 72 hours prior to addition of 0.5µCi of [³H]-thymidine (Amersham Biosciences, Amersham, UK) to each well. Wells with nTreg or iTreg only, Tresp only or unstimulated Tresp were used as controls. After 16 to 18

hours, incorporation of [^3H]-thymidine was measured using a liquid scintillation β -counter (Wallac, Turku, Finland) and expressed as mean counts per minute. Duplicate plates were used to measure cytokine levels at 72 hours of culture.

2.7. *In vivo* manipulations

2.7.1. Immunisations

Mice received 100 μg MOG₃₅₋₅₅ emulsified in complete Freund's adjuvant (CFA) containing 1mg/ml heat-killed *Mycobacterium tuberculosis* H37Ra (Sigma). A total volume of 100 μl was injected subcutaneously (s.c.) (50 μl into each hind limb).

2.7.2. CD4⁺ T cell transfers

Purified naïve CD4⁺ T cells, Th2 polarised cells, naïve and CNS-derived nTreg or *in vitro* generated iTreg were washed three times in sterile PBS and resuspended at the desired concentration. Cells were injected intravenously (i.v.) in a total volume of 200 μl .

2.7.3. *In vivo* CFSE-proliferation / suppression assays

CD4⁺ T cells were purified as described in section 2.5.4, resuspended at 5 $\times 10^7$ cells/ml in wash buffer and incubated with 500nM carboxyfluorescein succinimidyl ester (CFSE, Molecular Probes, Paisley, UK) per 10⁷ cells for 5 min at 37°C. Cells were washed twice in ice-cold 10% FCS complete media and counted. Cells were injected i.v. (2.7.2), with or without iTreg. This was followed by MOG₃₅₋₅₅/CFA immunization as described in 2.7.1. Unimmunized mice were used as controls.

2.7.4. Induction and assessment of EAE

Mice were immunised as in described in section 2.7.1. Doses of 2mg/ml or 4mg/ml CFA were used in some experiments as specified. Mice were given 200ng of pertussis toxin (PTX) (Health Protection Agency, Dorset, UK) in 500 μl PBS, intraperitoneally (i.p.) on the day of immunisation, repeated 2 days later. Clinical

signs of EAE were assessed daily from day 6 post-immunization, and scored as follows:

- 0 – No disease
- 1 – Flaccid tail
- 2 – Impaired righting reflex and/or abnormal gait
- 3 – Partial hind limb paralysis
- 4 – Total hind limb paralysis
- 5 – Partial forelimb paralysis
- 6 – Moribund or dead

Mice with grade 5 on two consecutive days and/or with the body weight loss exceeding 30% were euthanized.

2.7.5. Induction and assessment of rMOG-induced AAI

2.7.5.1. Intraperitoneal sensitisation

For the sensitization model of AAI, mice received two i.p. injections of 100µg or 200µg rMOG in an aqueous solution of aluminium hydroxide adjuvant at a 3:1 ratio (Imject Alum, Pierce, Rockford, USA) on day 0 and 14.

2.7.5.2. Th2 cell transfer

When airway inflammation was induced via Th2 cell transfer, mice received CD4⁺ sorted 5×10^6 Th2 polarised 2D2 cells i.v. in a total volume of 200 µl PBS on day 0.

2.7.5.3. Intratracheal instillation

Mice were anaesthetised with i.p. injection of ketamine (Fort Dodge Animal Health Ltd, Hampshire, UK) and medetomidine hydrochloride (Pfizer Ltd, Surrey, UK). A blunt cannula was gently introduced through the larynx into the trachea. A volume of 50µl of rMOG (50µg) in sterile PBS was injected intratracheally (i.t.). Anaesthesia was reversed by s.c. injection of antipamezole hydrochloride (Pfizer). Three i.t. injections were given 3 days apart: on days 21, 24 and 27 in the sensitisation AAI model, and on days 1, 4 and 7 in an adoptive Th2 cell transfer AAI model.

2.7.5.4. Bronchoalveolar lavage

Mice were sacrificed by i.p. injection of pentobarbitone. Bronchoalveolar lavage (BAL) was collected by cannulation of the trachea with a 27-gauge needle encased in 0.96-mm polyethylene tubing (Fanning Ltd, UK). Lungs were lavaged three times with 500µl PBS to obtain BAL fluid (BALF). BALF cells were counted, centrifuged at 350xg for 5 minutes, and supernatant was collected for the detection of cytokines. Cells were resuspended in PBS and cytopspins prepared using a Shandon Cytospin 3 centrifuge at 300rpm for 3 minutes. Slides were air dried for 20 minutes, fixed in ice-cold 100% methanol for 20 minutes and stained with Quick-Diff red stain for 1.5 minutes, followed by Quick-Diff blue stain for 15 seconds (both Gamidor Technical Services, Didcot, UK). Slides were rinsed in water and air dried. Differential cell counts were performed, blinded to experimental conditions, using light microscopy and a total of 200 cells per slide were counted.

2.7.5.5. Histological scoring

Lung were perfused through the right ventricle as described in 2.5.3, inflated and fixed in 10% formalin. Tissue was embedded in paraffin and sections were stained using hematoxylin and eosin (H&E) or Periodic Acid-Shiff (PAS) stains to detect mucus-producing cells (all provided by University of Edinburgh histology service). Inflammatory scoring of H&E stained slides was done manually at x200 magnification as previously described (Leech et al., 2007) Briefly, 10 consecutive fields were scored which included one complete blood vessel and one complete bronchiole (with diameters less than half of a field, equivalent to 300µm). Infiltration scores were applied to the peri-vascular and peri-bronchiolar compartments as follows:

- 1 - no cells
- 2 - less than 20 cells
- 3 - more than 20 cells but less than 100 cells
- 4 - more than 100 cells

The percentage of goblet cells in small airways (diameter ≤ 300 µm) and large airways (diameter >300 µm) was determined by calculating the average percentage

of goblet cells within 10 consecutive airways for small airways and as many as possible for large airways. All histological scoring was carried out blinded to experimental conditions.

2.8. Depletion of Foxp3⁺ cells using DTx

Foxp3.LuciDTR-4 were used which express eGFP, diphtheria toxin receptor (DTR) and luciferase under the *foxp3* promoter (Suffner et al., 2010). In this system administration of diphtheria toxin (DTx) enables selective depletion of Foxp3⁺ cells. A dose of 24ng/kg DTx was administered i.p. in a volume of 100µl on two consecutive days, and then every 3-4 days to maintain depletion. Blood was sampled every 2-4 days to determine depletion, using either GFP expression or intracellular Foxp3 expression.

2.9. Enzyme Linked Immunosorbent Assays

2.9.1. Cytokine quantification in cell supernatant

96-well plates (Maxisorp, Nunc, Rochester, USA) were coated with cytokine capture antibodies (Table 2.2) diluted in 1x bicarbonate buffer (Sigma) and incubated overnight at 4°C. Plates were washed twice in 1x PBS containing 0.1% Tween (PBS-Tween, Sigma) and blocked with 1% bovine serum albumin (BSA, Sigma) for 1 hour at 37°C. Plates were washed twice in PBS-Tween, loaded with cell supernatant and known cytokine standards, and incubated for 2 hours at room temperature. Standards were generated by performing doubling dilutions of recombinant mouse cytokines in 1% BSA-PBS. The sensitivity of cytokine assays was as follows; IFN-γ: 0.2-100ng/ml (BD Pharmingen, Oxford, UK), IL-17: 0.1-10ng/ml (R&D), IL-4: 0.01-5ng/ml (R&D), IL-13: 0.02-10ng/ml (eBioscience). Plates were washed 4 times in PBS-Tween prior to addition of biotinylated antibody (Table 2.2) diluted in 1% BSA-PBS for 1 hour at room temperature. Plates were washed 6 times in PBS-Tween and loaded with extravidin peroxidase (Sigma) diluted 1:1000 with 1% BSA-PBS. After 30 minutes of incubation at room temperature, plates were washed 6 times in PBS-Tween and the ELISA was developed by addition of phosphate-citrate buffer containing H₂O₂ and 100ng/ml tetramethyl-benzidine (TMB, Sigma). The

reaction was stopped by adding 2M sulphuric acid. Plates were read for optical absorbance at 450nm using a Biotek Synergy HT microplate reader (Winooski, USA) and analyzed by Gen5 software (Biotek)

GM-CSF was measured using ELISA development kit as per manufacturer's instructions: Mouse GM-CSF ELISA Ready-Set-Go (eBioscience). The sensitivity of the kit was 4-500pg/ml.

2.9.2. Measurement of rMOG-specific IgE in serum

Blood was taken via an axillary approach post-mortem, serum collected and frozen at -20°C for detection of rMOG specific IgE, IgG1 and IgG2a. For IgE detection, serum was diluted 1:4 with PBS and incubated with fast flow protein G sepharose beads (Sigma-Aldrich) overnight at 4°C on a rotator for the depletion of IgG, as described previously (Leech et al., 2007). 96-well plates (Maxisorp, Nunc) were coated with 5µg/ml rMOG in 0.05M carbonate buffer and incubated overnight at 4°C. Plates were blocked with 1% BSA-PBS for 1 hour at 37°C. Serum was centrifuged twice at 13000xg for 5 minutes to remove beads and diluted 1:10 in PBS. Serial dilutions of serum were prepared by performing doubling dilutions in 1% BSA-PBS. Plates were washed 5 times in 0.1% PBS-Tween, loaded with duplicates of pre-diluted serum, and incubated overnight at 4°C. Plates were washed 5 times in PBS-Tween and biotinylated rat anti-mouse IgE antibody (clone R35-118, BD Bioscience) at 2µg/ml was added to each well. Plates were incubated for 1 hour at 37°C and washed 5 times in PBS-Tween prior to addition of extravidin peroxidase (Sigma), diluted 1:200 with 1% BSA-PBS. After 1 hour incubation at room temperature, plates were washed 6 times in PBS-Tween and the ELISA was developed by the addition of phosphate-citrate buffer containing H₂O₂ and 100ng/ml tetramethyl-benzidine (TMB, Sigma). The reaction was stopped by adding 2M sulphuric acid. Since no standard was used, each plate was developed for exactly the same time prior to stopping the reaction. Plates were read for optical absorbance at 450nm using a Biotek Synergy HT microplate reader (Biotek) and analyzed using Gen5 software (Biotek).

2.9.3. Measurement of rMOG-specific IgG1 and IgG2a in serum

96-well plates (Maxisorp, Nunc) were coated with 5µg/ml rMOG in 0.05M carbonate buffer and incubated overnight at 4°C. Plates were blocked with 3% BSA-PBS for 1 hour at 37°C. Serum was diluted 1:5 in PBS-Tween and doubling dilutions were prepared. Plates were washed 5 times in 0.1% PBS-Tween, loaded with duplicates of pre-diluted serum and incubated for 1 hour at 37°C. Plates were washed 5 times in PBS-Tween and detection antibody diluted in PBS-T was added to each plate: goat anti-mouse IgG1AP and IgG2aAP (both 1:500) (Southern Biotech, Birmingham, USA). Plates were incubated for 1 hour at 37°C and washed 5 times in PBS-Tween. The ELISA was developed by addition of pNNP (Southern Biotech) in a substrate buffer containing 0.24mM magnesium chloride hexahydrate and 96mM diethanolamine (Sigma) and the plates were read at 405 nm (Anthos ELISA reader, Biochrom Ltd, Cambridge, UK). As no standard was used, each plate was developed for exactly the same time prior to reading.

2.10. Cytokine analysis using 13-plex Flowcytomix Multiplex

Mouse Th1/Th2/Th17/Th22 13plex FlowCytomix Multiplex kit (eBioscience) was used to measure cytokines in BALF as per manufacturer's instructions. Briefly, a mixture of antibody-coated beads and a mixture of secondary antibodies were added to 25µl of each sample and incubated at room temperature for 2 hours on a microplate shaker at 500rpm. Followed by two washes, Streptavidin- Phycoerythrin solution was added and incubated at room temperature for 1 hour. Followed by two washes, the samples were analyzed by flow cytometry. Different bead sizes and intensities of conjugated dye enabled differentiation between different cytokines. The use of standard allowed for a calculation of cytokine concentration in a sample.

2.11. Immunohistochemistry

Slides were placed for 15 minutes at 59°C to soften the paraffin, then deparaffinized by sequential immersion in the following: xylene for 10 min, absolute alcohol for 40 seconds, 95% alcohol for 20 seconds, 70% alcohol for 20 seconds, then washed in

water for 1 minute. Next, they were mounted onto Shandon coverplates (ThermoFisher Scientific, Waltham, USA) and put into a Shadon Sequenza Slide Rack. The slides were blocked with 3% hydrogen peroxide solution for 30 minutes at room temperature and washed once with wash buffer containing 0.05M Tris-HCl, 0.15M NaCl and 0.05% Tween (all from Sigma). Next, they were blocked with avidin D solution (Vector Laboratories Ltd, Peterborough, UK) in 1.5% Normal Rabbit Serum (NRS) for 20 minutes at room temperature and washed once in wash buffer. The rat anti-mouse Major Basic Protein monoclonal antibody (clone MT-14.7, provided by Dr James Lee, Mayo Clinic, USA) was used at 1 in 500 in 1.5% NRS and added to slides for 40 minutes at room temperature. Rat IgG1 κ antibody (Vector Laboratories Ltd) was used as an isotype control. The slides were washed 3 times in wash buffer and incubated with a secondary anti-rat IgG-Biotinylated mouse adsorbed antibody (Vector Laboratories Ltd) for 30 minutes at room temperature. The slides were then washed, and Streptavidin-HRP (Vector Laboratories Ltd) was added at 1:1000 for 1 hour at room temperature. Following a washing step, DAB reagent (Vector Laboratories Ltd) was added for 10 minutes at room temperature, slides were counterstained with 0.1% methyl green (Sigma) and mounted with VectaMount (Vector Laboratories Ltd). The staining was visualized using light microscopy.

2.12. Flow cytometric analysis

2.12.1. Antibodies for flow cytometric analysis

Antibodies used for flow cytometry were from eBioscience unless otherwise stated and are summarised in Table 2.1.

2.12.2. Viability staining

Where stated, cells prior to surface staining were washed twice in PBS, stained with fixable viability dye eFluor780 (eBioscience) at 1:1000 in a total volume of 500 μ l PBS for 20 minutes at 4°C. Cells were then washed in FACS buffer.

2.12.3. Surface staining

Cells were washed in FACS buffer and stained with the indicated antibodies (see Table 2.1) in 50µl FACS buffer for 20 min at 4°C. After washing in FACS buffer, cells were either resuspended in FACS buffer for immediate analysis, or fixed in 1% paraformaldehyde (PFA) and stored at 4°C for next day analysis.

2.12.4. Intracellular cytokine staining

Cells were incubated with Brefeldin A (eBioscience) for the final 4 to 5 hours of culture. Where stated, cells were also incubated with 50ng/ml phorbol myristate acetate (PMA) and 1µg/ml ionomycin (both from Sigma) for the same amount of time. Cells were washed in FACS buffer and, where stated stained with viability dye (eBioscience, see section 2.12.2). Cells were stained for cell surface markers as described in 2.12.3, resuspended in 200µl of BD Cytotfix/Cytoperm solution (BD Pharmingen) and incubated for 20 minutes at 4°C. Cells were washed twice in 1x BD Perm/Wash buffer (BD Pharmingen) and stained for intracellular cytokines using the indicated antibodies (Table 2.1) diluted in 50µl Perm/Wash buffer (BD Pharmingen) at 4°C for 30 minutes. Cells were washed in Perm/Wash (BD Pharmingen) buffer and resuspended in FACS buffer for immediate or next day analysis. Alternatively, in earlier experiments, after cell surface staining cells were stained for intracellular cytokines using Foxp3 Fixation/Permeabilisation buffers from eBioscience as described below for intracellular staining for transcription factors (2.12.5).

2.12.5. Intracellular staining for transcription factors

Cells were surface-stained as described in 2.12.3 and resuspended in 400µl Foxp3 Fixation/Permeabilisation buffer (eBioscience). After overnight incubation at 4°C, cells were washed in FACS buffer and stained with the indicated antibodies (Table 2.1) diluted in 50µl of 1×Permeabilisation buffer (eBioscience) for 30 min at 4°C. Cell were then washed and resuspended in FACS buffer for immediate or next day analysis.

2.12.6. Flow cytometric data analysis

Flow cytometric data was acquired using a Becton Dickinson (Franklin Lakes, USA) LSRII Fortessa II and analysed using FlowJo software (Tree Star version 3.2.1, Ashland, USA).

2.13. Statistics

All statistics was performed using GraphPad Prism software (San Diego, USA). Unpaired t-test or Mann-Whitney U test were used when comparing two groups. To compare three and more groups a one-way analysis of variance (ANOVA) was used with Tukey's multiple comparison post test. Alternatively, an unpaired t-test was used to test differences between the groups of interest. The Kruskal-Wallis test with Dunn's multiple comparison post test was used to compare three or more groups where data were non-parametric. Bonferroni's post test was used for analysis of ELISA data. Due to the ranked nature of EAE disease scores, a Fisher's exact test was used to determine differences in disease severity (i.e. the number of mice with a score ≤ 2 , versus the number of mice with a grade ≥ 3). Data were considered statistically different with p values of <0.05 .

Antibody	Conjugate	Clone	Final concentration
CD3	PerCP-Cy5.5	145-2C11	1µg/ml
CD4	AlexaFluor700 [§] eFluor450, PerCP	RM4-5 RM4-5	2µg/ml 1µg/ml
CD45.1	PE [^] , PerCP-Cy5.5	A20	1µg/ml
CD45.2	APC	104	1µg/ml
CD90.1	APC	HIS-51	1µg/ml
CD90.2	PE [^]	53-2.1	1µg/ml
Vα3.2	FITC [^] , PE*	RR3-16	1µg/ml
Vβ11	Biotinylated	CTVB11	1µg/ml
CD11b	APC, eFluor450	M1/70	1µg/ml
CD62L	PE, Biotinylated [^]	MEL-14	2.5µg/ml
CD44	APC-Cy7*	IM7	1µg/ml
CD73	PE	Ty/11.8	1µg/ml
CXCR3	APC	CXCR3-173	5µg/ml
T-bet	PerCP-Cy5.5	eBio4B10	1µg/ml
GATA-3	PE	TWJ	0.6µg/ml
Foxp3	FITC eFluor450	FJK-16s FJK-16s	5µg/ml 2µg/ml
IFN-γ	APC, FITC, PE [^]	XMG1.2	2µg/ml
IL-17A	PerCP-Cy5.5	eBIO17B7	2µg/ml
TNF-α	eFluor450	MP6-XT2	2µg/ml
GM-CSF	PE [^]	MP1-22E9	4µg/ml
IL-13	AF647	eBio13A	2µg/ml
IL-5	PE	TRFK5	2µg/ml
IgG1	FITC, eFluor450, PE, AF647 [^] PerCPCy5.5*	eBRG1 eBRG1 P3.6.2.8.1	2µg/ml 2µg/ml 1µg/ml
IgG2a	FITC, eFluor450 Biotinylated, PE, PerCP-Cy5.5	eBR2a eBR2a MOPC-173	5µg/ml 2.5µg/ml 2µg/ml
IgG2b	PE [^] APC-Cy7*	27-35 RTK4530	0.6µg/ml 1µg/ml
Armenian hamster IgG	APC	HTK888	5µg/ml
Streptavidin	PerCP-Cy5.5 [^]	N/A	2µg/ml

Table 2.1 Antibodies used for flow cytometry.

All from eBioscience unless otherwise stated. *BioLegend, [^]BD Biosciences, [§]Invitrogen.

Capture antibody (purified)	Clone	Final concentration	Detection antibody (biotinylated)	Clone	Final concentration
anti-IFN-γ	R4-6A2	2µg/ml	anti-IFN-γ	XMG1.2	0.5µg/ml
anti-IL-17A	TC11-18H10	0.5µg/ml	anti-IL-17A	TC11-8H4.1	0.25µg/ml
anti-IL-13	eBio13A	2µg/ml	anti-IL-13	eBio1316H	0.5µg/ml
anti-IL-4	11B11	2µg/ml	anti-IL-4	BVD6-24G2	1µg/ml

Table 2.2 Capture and detection antibodies used for cytokine ELISA.

All from BD Pharmingen apart from anti-IL-13 antibodies which were obtained from eBioscience.

3 Developing a model of rMOG-induced allergic airways inflammation

3.1. Introduction

One of the main aims of this project was to determine whether Treg can acquire a different phenotype in Th1/Th17-mediated autoimmune CNS inflammation in EAE and in Th2-mediated allergic lung inflammation in AAI and if so, to test how this affects their suppressive capacity. It has been previously demonstrated that antigen reactivity is important for an effective Treg-mediated inhibition of CD4⁺ T cell responses both *in vitro* and *in vivo* (Thornton and Shevach, 2000, Tang et al., 2004). In a model of MOG₃₅₋₅₅-induced EAE it was shown that CNS-derived Treg have a superior capacity to suppress disease severity over polyclonal Treg (McGeachy et al., 2005) and an increased frequency of myelin-specific Treg was reported in the inflamed CNS of mice in a self-resolving model of EAE (O'Connor et al., 2007, Korn et al., 2007). Moreover, therapeutic transfers of Treg demonstrated that only antigen-reactive and not polyclonal Treg were capable of suppressing EAE (Stephens et al., 2009). In models of AAI it has been demonstrated that polyclonal Treg effectively inhibit disease (Kearley et al., 2005, Leech et al., 2007). However, no study has directly compared the suppressive efficiency of antigen-reactive versus polyclonal Treg in the context of this disease.

Hence, in order to distinguish between the effect of a different Treg phenotype and different antigen responsiveness it was decided that Treg of the same antigen reactivity would have to be transferred from a Th1-mediated inflammatory disease into a Th2-driven disease, and vice versa. For this to be achieved, a new model of autoantigen-mediated AAI had to be developed as the CNS inflammation in EAE can only be driven by a myelin-derived self-peptide. Extracellular domain of MOG, recombinant MOG (rMOG), has been shown to effectively induce EAE (Fillatreau et al., 2002). Previously in the Anderton lab it was demonstrated that sensitisation of C57BL/6 mice with rMOG in alum resulted in an increased level of Th2-associated rMOG-specific IgG1 antibody in serum (Mel Leech, unpublished observations). For

this reason it was decided to use rMOG as an allergen to establish a new model of Th2-driven AAI.

3.1.1. Aims

The aims of the experiments described in this chapter were to:

- 1) Induce AAI in C57BL/6 mice using rMOG.
- 2) Characterize the disease and immunological readouts of this model.
- 3) Establish disease kinetics and recovery time.
- 4) Assess the importance of Foxp3⁺ Treg in the control of lung inflammation in this model.

3.1.2. Experimental approach

The first approach was to develop an rMOG-induced AAI model in C57BL/6 mice using standard alum sensitization and airway challenge protocols (Kearley et al., 2005, Leech et al., 2007). In the light of possible self-tolerance mechanisms, MOG-deficient mice (Delarasse et al., 2003) were used to test whether their lack of endogenous MOG expression would make them more susceptible to developing rMOG-induced AAI. A different approach to induce AAI was also taken that involved an adoptive transfer of traceable activated MOG-reactive CD4⁺ T cells isolated from a 2D2 mouse (Bettelli et al., 2003) which were polarised *in vitro* towards Th2 phenotype. These were transferred into naïve mice followed by antigen airway challenge. To investigate changes within Foxp3⁺ cell population, Foxp3-GFP mice (Fontenot et al., 2005b) were used in some experiments as it enabled identification of Treg without the need for intracellular Foxp3 staining. Foxp3.LuciDTR4 mice (Suffner et al., 2010), in which administration of DTx allows for selective depletion of Foxp3⁺ cells, were used to investigate the importance of Foxp3⁺ Treg in a model of rMOG-induced AAI.

3.2. Results

3.2.1. Sensitization with rMOG in alum and subsequent airway challenges do not induce AAI in C57BL/6 mice

A dose of antigen in alum required to sensitize wild-type (WT) C57BL/6 mice varies according to the protocol and allergen used for an induction of AAI, and is typically within a range of 10 μ g to 100 μ g (Leech et al., 2007, Zhang et al., 2009, Mackenzie, 2011). In addition to Th2-associated IgE, immunoglobulin isotypes IgG2a and IgG1 are commonly used as markers of Th1 and Th2 responses, respectively (Mosmann et al., 1986, Snapper and Paul, 1987). In order to establish an effective sensitization dose of rMOG in alum, C57BL/6 mice were given two intraperitoneal injections of 100 μ g or 200 μ g of rMOG adsorbed to alum 14 days apart and serum was analysed on day 21 for the presence of rMOG-specific IgG2a and IgG1. rMOG-specific IgG1 but not IgG2a was detected in serum of mice sensitised with 200 μ g but not with 100 μ g of rMOG (data not shown), and hence this dose was chosen to be used in the AAI induction protocol.

To induce AAI, C57BL/6 mice were given two intraperitoneal injections of 200 μ g of rMOG in alum followed by three intratracheal challenges with 50 μ g of rMOG or PBS as a control (Figure 3.1A). In order to trace antigen-reactive T cells, naïve 2D2 CD4⁺ cells, expressing TCR recognizing the 35-55 peptide of MOG protein (Bettelli et al., 2003), were transferred intravenously prior to sensitization phase in one experimental group. Bronchoalveolar lavage (BAL) fluid was collected to prepare cytopspins for differential BAL counts and the whole lung was perfused with 10% formalin to be used for histology or left unperfused for flow cytometric analysis. Sensitization and airway challenges with rMOG were not found to affect total cell numbers in BAL fluid (Figure 3.1B) and although the presence of eosinophils was more pronounced in rMOG-challenged groups the increase compared to PBS-challenged mice was not significant (Figure 3.1C). The cellular infiltration of the lung was quantified using a previously published inflammatory scoring system (Leech et al., 2007). The infiltration of the blood vessels and bronchioles was very low or absent in rMOG-challenged mice and not significantly different from the control mice (Figure 3.1D&E). Period Acid Schiff (PAS) staining which detects

mucus produced by goblet cells revealed low percentages of goblet cells in small and large airways of rMOG-challenged mice without 2D2 cell transfer, and in large airways of those mice that received 2D2 cells (Figure 3.1D&F). In order to confirm that the mice were sensitized, serum levels of rMOG-specific IgE, IgG2a and IgG1 were tested using ELISA. Both IgE and IgG1 were detected in serum of mice sensitized and airway challenged with rMOG (Figure 3.2A&B) and IgG2a was undetectable (Figure 3.2C). Transfer of 2D2 cells did not enhance antibody titers.

Flow cytometric analysis of ex vivo 2D2 cells from the lung, mediastinal lymph nodes (medLN) and spleen of rMOG-challenged mice showed that 2D2 cells became activated in response to antigen *in vivo* but their low total cell numbers indicated poor expansion and/or survival (data not shown). It was concluded that sensitisation and airway challenges with rMOG in C57BL/6 mice do not result in an allergic lung inflammation.

3.2.2. MOG-deficient mice develop eosinophilia but not lung inflammation upon sensitization and challenge with rMOG

As already mentioned, a possible explanation for the failure of rMOG to induce Th2-mediated AAI in WT C57BL/6 mice could be the fact that numerous tolerance mechanisms towards self-antigens have evolved to limit autoimmune reactions (section 1.3). To test this hypothesis it was decided to use MOG-deficient mice in which the gene encoding MOG protein is disrupted (Delarasse et al., 2003). These mice present no clinical or histological abnormalities otherwise. In this system MOG should be considered a foreign antigen. To induce AAI, MOG-deficient mice were given two intraperitoneal injections of 100µg of rMOG in alum followed by three intratracheal challenges with 50µg of rMOG or PBS as a control (Figure 3.3A).

Total BAL cells were not significantly different between rMOG- and PBS-challenged groups (Figure 3.3B) however a significant increase in the percentage of eosinophils in BAL was noted upon antigen challenge, accounting for ~35% of total BAL cells (Figure 3.3C). Eosinophils constituted less than 1% of total BAL cells in

the control group where the majority of cells were alveolar macrophages. Low inflammatory infiltrates around the blood vessels and bronchioles were observed in both rMOG- and PBS-challenged groups. (Figure 3.3 D&E). The percentages of goblet cells were very low and similar in both groups in large airways and present in small airways only in the rMOG-challenged group (Figure 3.3D&F). rMOG-specific IgE and IgG1 but not IgG2a were detected in serum of both rMOG- and PBS-challenged mice and were not significantly different (Figure 3.4A,B&C).

Flow cytometric analysis of the lung, medLN and spleen revealed that total cell numbers across organs were similar in rMOG- and PBS-challenged mice (data not shown). The percentages and total numbers of CD4⁺ T cells were also unchanged (Figure 3.5A&B) however there was a significant increase in the frequency and total number of Foxp3⁺ CD4⁺ cells specifically within the lung of rMOG-challenged mice (Figure 3.5C&D). Although MOG-deficient mice displayed better allergic response to MOG and developed lung eosinophilia, this AAI model was considered not to be robust enough for investigating the role and therapeutic potential of Treg. A different approach was adopted which involved a transfer of antigen-reactive CD4⁺ T cells polarised *in vitro* towards Th2 phenotype prior to antigen airway challenge.

3.2.3. Optimisation of Th2 polarising protocol of MOG₃₅₋₅₅-reactive cells.

To develop an optimal protocol for polarising pure Th2 cells *in vitro*, IL-4-GFP CD4⁺ cells, which express GFP under the *il4* promoter, were studied. IL-4-GFP whole splenocytes were stimulated with soluble α -CD3 for 3 days in the presence of Th1-inducing cytokines (IL-12 and IL-18) or Th2-inducing conditions (IL-4, α -IL-12, α -IFN- γ) with or without IL-2 (Figure 3.6A). The presence of IL-4, α -IL-12 and α -IFN- γ resulted in IL-4 production in approximately 16% of CD4⁺ cells and a specific upregulation of Th2 master regulator GATA-3 (data not shown). Addition of exogenous IL-2 greatly enhanced cell survival and promoted GATA-3 expression and expansion of IL-4-secreting CD4⁺ cells to approximately 28% (Figure 3.6B&C). IL-4⁺ cells, but very low GATA-3 expression, were also detected in Th1 conditions which could be explained by non-specific IL-4 upregulation during the first few days

of polarisation (Figure 3.6B&C). T-bet expression was specific to Th1 cells and was very low in Th2 cells (Figure 3.6C). The extent of Th2 polarisation was also tested by cytokine assays which detected higher levels of IL-13 and IL-5 in Th2-polarised cell culture supernatants versus Th1 supernatants (Figure 3.6D). In order to assess whether contaminating IFN- γ^+ cells were present in Th2 cultures and to verify Th2 polarisation protocol in a TCR transgenic system, MOG₃₅₋₅₅-reactive 2D2 cells were stimulated for 3 days under polarising conditions and stained for the intracellular expression of IFN- γ and IL-13. Approximately 11-15% of 2D2 CD4 $^+$ T cells polarised under Th2 conditions were IL-13 $^+$ and 1% was IFN- γ^+ compared to less than 1% of IL-13 $^+$ and approximately 47% of IFN- γ^+ cells generated under Th1 polarising conditions (Figure 3.6E). Less than 1% of double producers was detected in each condition (Figure 3.6E).

It was concluded that IL-2 boosts Th2 polarisation and 3-day stimulation under polarising conditions *in vitro* allows for enough time to obtain Th2 cells.

3.2.4. Adoptive transfer of Th2 polarised 2D2 CD4 $^+$ cells followed by airway challenge induces AAI

In order to assess the potential of Th2 polarised 2D2 CD4 $^+$ cells to infiltrate the lung and induce airway inflammation, 5×10^6 of traceable CD4 $^+$ sorted Th2 polarised 2D2 cells were transferred into WT hosts followed by three consecutive intratracheal injections with rMOG or PBS as a control (Figure 3.7A). The number of transferred cells was based on previous reports which used Th2 polarised OVA-reactive CD4 $^+$ T cells to induce an OVA-mediated model of AAI (Cohn et al., 1997, Hogan et al., 1998). Th2 polarisation of 2D2 cells on the day of transfer was confirmed by ELISA for IL-13 and IL-5 and GATA-3 staining (data not shown). Analysis of the cellular composition of BAL and lung tissue revealed that the greatest evidence for inflammation was on day 9 (Appendix 1&2). This time point was therefore chosen for further detailed investigation of the inflammatory and CD4 $^+$ cell response.

Two days after the third intratracheal injection the left lobe of the lung, medLN and the spleen were harvested and analysed by flow cytometry. BAL fluid was collected

to prepare cytospins for differential BAL counts and the two right lobes of the lung were perfused with 10% formalin and used for histology. BAL analysis revealed that the total cell number was significantly increased in mice challenged with rMOG (Figure 3.7B). The percentages of cell types varied between the experiments and the average contribution of eosinophils from pooled experiments was approximately 25-30%, macrophages 70-75% and lymphocytes 2-3% (Figure 3.7C&E). The frequency of neutrophils was negligible. Eosinophils constituted less than 1% of total BAL cells in the control group where the majority of cells were alveolar macrophages (Figure 3.7C&E). The absolute numbers of eosinophils, macrophages and lymphocytes were significantly increased in the rMOG-challenged group (Figure 3.7D, F&G). Cytokine bead array analysis of BAL fluid detected Th2-associated IL-13 and IL-5 and low levels of IL-4 and IL-10 (Figure 3.8). No IFN- γ or IL-17 was found.

In the lung tissue itself, significantly increased cellular infiltrates were detected around the blood vessels and bronchioles of rMOG-challenged mice (Figures 3.9A&B). Staining for major basic protein, a major component of secretory vesicles in eosinophils, revealed that eosinophils substantially constituted to the lung infiltrate (Figure 3.9D). PAS staining revealed that in rMOG-challenged mice ~12% of small airway epithelial cells and ~30% of large airway epithelial cells were goblet cells (Figure 3.9A&C). Control mice had negligible percentage of goblet cells in small airways and approximately 5% in large airways (Figure 3.9A&C). Naïve 2D2 CD4⁺ CD62L^{high} CD44⁻ cells were used as an additional control in some experiments and were not found to induce lung eosinophilia and infiltration (data not shown). Moreover, 2D2 cells polarised towards Th2 phenotype for 4 to 6 days were also compared for their ability to induce lung inflammation and were found to give comparable results to inflammation driven by 2D2 cells polarised for 3 days (data not shown).

3.2.5. rMOG-induced AAI is characterized by increased frequency of Th2 2D2 cells within the lung and medLN

The analysis of total cell numbers revealed significantly increased cell numbers in the lung and medLN and no major changes in the spleen cellularity upon rMOG

challenge (Figure 3.10B). Using flow cytometry the differences in the percentages of total CD4⁺ cells and donor Th2 2D2 cells were investigated. The percentage of total CD4⁺ cells was significantly increased in the lung in response to rMOG challenge from approximately 10% to 14% and no significant changes were detected in the medLN and spleen where the CD4⁺ cell percentages varied between 19% and 25% (Figure 3.10C). The total numbers of CD4⁺ cells followed the same pattern and were only significantly increased in the lung tissue (data not shown).

Looking at the transferred Th2 2D2 cells, their percentages and absolute cell numbers were significantly increased within the lung and medLN of rMOG-challenged mice and were unchanged in the spleen compared to PBS-challenged mice (Figure 3.11 A&B). In the lung the percentage of Th2 2D2 cells was ~11% and in the medLN ~15% compared to 2% observed in the spleen (Figure 3.11A). The percentage of 2D2 cells in uninvolved lymph nodes from rMOG-challenged mice was found to be very low (data not shown). In terms of mean total cell numbers, a ~9-fold increase in the lung and a ~70-fold increase in the medLN were noted upon rMOG challenge whereas numbers in the spleen were not altered (Figure 3.11B). This suggests a requirement for the *in vivo* presence of antigen in the migration / survival / proliferation of activated Th2 2D2 cells.

3.2.6. Th2 2D2 cells have a stable phenotype *in vivo* following rMOG challenge

Th2 2D2 cells prior to transfer expressed high levels of GATA-3 and produced IL-13 and IL-5 as determined by ICS and ELISA (Figure 3.6E, data not shown). In order to determine how stable this phenotype was *in vivo* after transfer, the cells from the lung, medLN and spleen were stained for the intracellular expression of GATA-3 and analysed by flow cytometry. Th2 2D2 cells from the rMOG-challenged lung retained higher expression of GATA-3 compared to Th2 2D2 cells isolated from the PBS-challenged control (Figure 3.12A). Moreover, within the rMOG-challenged group, Th2 2D2 cells from the lung retained the highest GATA-3 expression versus the cells from the medLN and the spleen (Figure 3.12A&B). This organ-specific elevation in GATA-3 expression was not noted in host CD4⁺ cells.

Cells isolated from the lung, medLN and spleen were re-stimulated overnight with MOG₃₅₋₅₅ followed by intracellular staining for IL-13. The lung and medLN of PBS-challenged mice did not have sufficient numbers of Th2 2D2 cells for re-stimulation assays and the data from the spleen are not shown. On average approximately 7% of Th2 2D2 cells from the rMOG-challenged lung produced IL-13 which was significantly higher compared to Th2 2D2 cells isolated from the rMOG-challenged medLN and spleen (Figure 3.13A&B). The percentages of IL-13⁺ Th2 2D2 cells from the medLN were very low (>0.5%) and from the spleen they reached ~2% (Figure 3.13B). IFN- γ production by Th2 2D2 cells within the lung was investigated in one of the experiments and its value from 4 pooled mice was found to be below 1.5% which is similar to IFN- γ production by Th2 2D2 cells on the day of transfer (data not shown). These results suggest that the antigen challenge and the inflammatory environment of the lung maintain the Th2 phenotype of 2D2 cells.

3.2.7. Percentage and total number of Foxp3⁺ cells is elevated in the lungs of rMOG-challenged mice

It was previously reported that Foxp3⁺ CD4⁺ cells accumulate in the lung and medLN during lung inflammation in a murine model of HDM-induced AAI involving alum sensitization (Leech et al., 2007). To test whether the same phenomenon occurs in rMOG-induced AAI using an adoptive transfer of Th2 2D2 cells, the cells were stained for the intracellular expression of Foxp3. A significant increase in the percentages of total Foxp3⁺ CD4⁺ cells from 6% to 10% was observed in the lung of rMOG-challenged mice and this translated into a ~3.5-fold increase in the total Foxp3⁺ cell numbers (Figure 3.14A&B). The percentages of Foxp3⁺ CD4⁺ cells were unchanged in the medLN (~10-13%) however the total numbers increased ~7-fold (Figure 3.14A&B) as the medLN in rMOG-challenged mice were significantly more cellular (Figure 3.10B). The percentages (~13%) and total numbers of Foxp3⁺ CD4⁺ cells in the spleen were unchanged (Figure 3.14A&B).

3.2.8. DTx administration protocol for depletion of Foxp3⁺ cells during AAI

To assess the importance of Foxp3⁺ CD4⁺ cells in the control of lung inflammation in rMOG-induced AAI, Foxp3⁺ cells were depleted prior to and throughout the duration of the disease. Transgenic Foxp3.LuciDTR-4 mice were used in which administration of diphtheria toxin (DTx) allows for selective ablation of Foxp3⁺ cells by utilizing the human diphtheria toxin receptor (DTR) system (Suffner et al., 2010). Moreover, Foxp3⁺ cells can be easily detected in this system by their expression of eGFP. The DTx treatment protocol was adapted from the study by Suffner et al. who observed that two doses of DTx on consecutive days are required for ~90-95% depletion of Foxp3⁺ cell compartment within 24 hours after the second treatment. As the effect of DTx was shown to wear off, additional DTx injections on days 2, 5 and 8 were performed during AAI induction (Figure 3.15A). It has to be noted that in Foxp3.LuciDTR-4 mouse ~5-10% of Foxp3⁺ cells do not have DTR and hence Foxp3⁺ cells surviving DTx treatment can rapidly reconstitute the Foxp3⁺ cell compartment by 2 weeks (Suffner et al., 2010).

In order to determine the extent of Foxp3⁺ cell depletion during AAI, the blood of DTx- and PBS-treated mice was sampled prior to the treatment and on days 2, 5 and 9 of the AAI protocol. Foxp3⁺ cell ablation was determined by both eGFP expression and by intracellular staining for Foxp3 expression. Due to the Foxp3 staining requirements, simultaneous analysis of eGFP and Foxp3 expression was not possible. This is due to the fact that Foxp3 staining requires a permeabilisation/fixation step which leads to eGFP bleaching making it undetectable. Two doses of DTx on days -2 and -1 were shown to deplete ~99.4% of eGFP⁺ cells as checked 24 hours after the second treatment (Figure 3.15B). The Foxp3 staining could not be performed on this day due to simultaneous Th2 2D2 cell transfer and hence insufficient amount of blood taken for both analyses. Blood analysis 72 hours after DTx treatment (d2) and prior to the third DTx injection revealed depletion of ~95.4% of eGFP⁺ cells and ~51.3% of Foxp3⁺ CD4⁺ cells (Figure 3.15B). Whereas, additional doses of DTx on days 2, 5 and 8 resulted in ~84.7% and ~95.4% depletion of eGFP⁺ cells as checked on day 5 and 9, the analysis of Foxp3 staining revealed no significant differences in the percentages of Foxp3⁺ CD4⁺ cells on these days (Figure 3.15B). These results

suggest that Foxp3⁺ cells were significantly depleted at the start of AAI induction and recovered in blood by day of the second antigen challenge.

3.2.9. Host Foxp3⁺ CD4⁺ cells are important in controlling rMOG-induced AAI

Following the depletion of host Foxp3⁺ cells prior to and during induction of AAI increased total cell numbers in BAL of DTx-treated mice were noted irrespective of challenge, however they were not significantly elevated when tested against PBS-treated groups (Figure 3.16A). The percentage composition of BAL revealed no significant changes in the eosinophil or lymphocyte contribution upon DTx treatment (Figure 3.16B). Total numbers of eosinophils were low in the PBS-treated rMOG-challenged group in this experiment and were not significantly affected by DTx treatment (Figure 3.16C). Total numbers of alveolar macrophages were significantly higher in the DTx-treated PBS-challenged group (Figure 3.16D) and no changes in the low neutrophil counts were observed (Figure 3.16E). Total numbers of lymphocytes were not significantly elevated in any of the groups, however they seemed to be higher in the DTx-treated rMOG-challenged group (Figure 3.16F).

In terms of lung pathology, both peri-vascular and peri-bronchiolar infiltration scores were significantly higher in the DTx-treated rMOG-challenged group compared to the PBS-treated group (Figure 3.17A&B). Percentages of goblet cells in small and large airways were low in the PBS-treated rMOG-challenged group and were not significantly affected by DTx treatment (Figure 3.17C).

The total cell numbers in the lung and medLN were not significantly changed by DTx treatment (Figure 3.18A). However, the percentages of CD4⁺ cells were significantly increased in the lung upon DTx treatment in both rMOG- and PBS-challenged mice (Figure 18B). In the medLN, the frequency of CD4⁺ cells was significantly decreased in DTx-treated rMOG-challenged mice (Figure 18B). The total cell numbers and percentages of CD4⁺ cells in the spleen were unaffected (Figure 18A&B). The percentages and total numbers of Th2 2D2 cells in the lung and medLN were not significantly affected by DTx treatment (Figure 3.19A&B).

The ability of lung-derived Th2 2D2 cells to produce Th2-associated cytokines was assessed by re-stimulating them overnight with MOG₃₅₋₅₅. In rMOG-challenged DTx-treated mice, significantly higher percentages of IL-13⁺ and GM-CSF⁺ Th2 2D2 cells were detected compared to the PBS-treated group (Figure 3.20). Similar pattern of results was found in the spleen (data not shown). These results collectively indicate that depletion of Foxp3⁺ cells by DTx treatment enhances certain disease parameters of rMOG-induced AAI.

3.2.10. Depletion of host Foxp3⁺ cells is associated with increased frequency of Foxp3⁺ Th2 2D2 cells

In order to determine the efficiency of Foxp3⁺ cell depletion in the lung and medLN at the time of harvest, the cells were stained for the intracellular expression of Foxp3. The percentages and the absolute numbers of total Foxp3⁺ cells were not significantly affected by DTx treatment in neither the lung, medLN nor the spleen (Figure 21A&B). The percentage of host Foxp3⁺ cells was found to be unaffected in the lung (data not shown). However, it was significantly reduced in the medLN of both DTx-treated groups (Figure 3.21C). The total numbers of host Foxp3⁺ cells in the medLN were, however, comparable to the PBS treated controls (Figure 3.21C).

To investigate what accounted for the unchanged percentage of total Foxp3⁺ cells and decreased percentage of host Foxp3⁺ cells in the medLN, Foxp3 expression was assessed in the transferred Th2 2D2 cells (Figure 3.22A). On the day of transfer, Foxp3⁺ cells constituted less than 1% of Th2 2D2 cells (data not shown). The percentages of Foxp3⁺ Th2 2D2 cells were significantly increased specifically within the medLN and spleen of DTx-treated rMOG-challenged mice (Figure 3.22A&B). The total number of Foxp3⁺ Th2 2D2 cells within the medLN, lung and spleen were not significantly affected however they seemed to be elevated (Figure 3.22A&B).

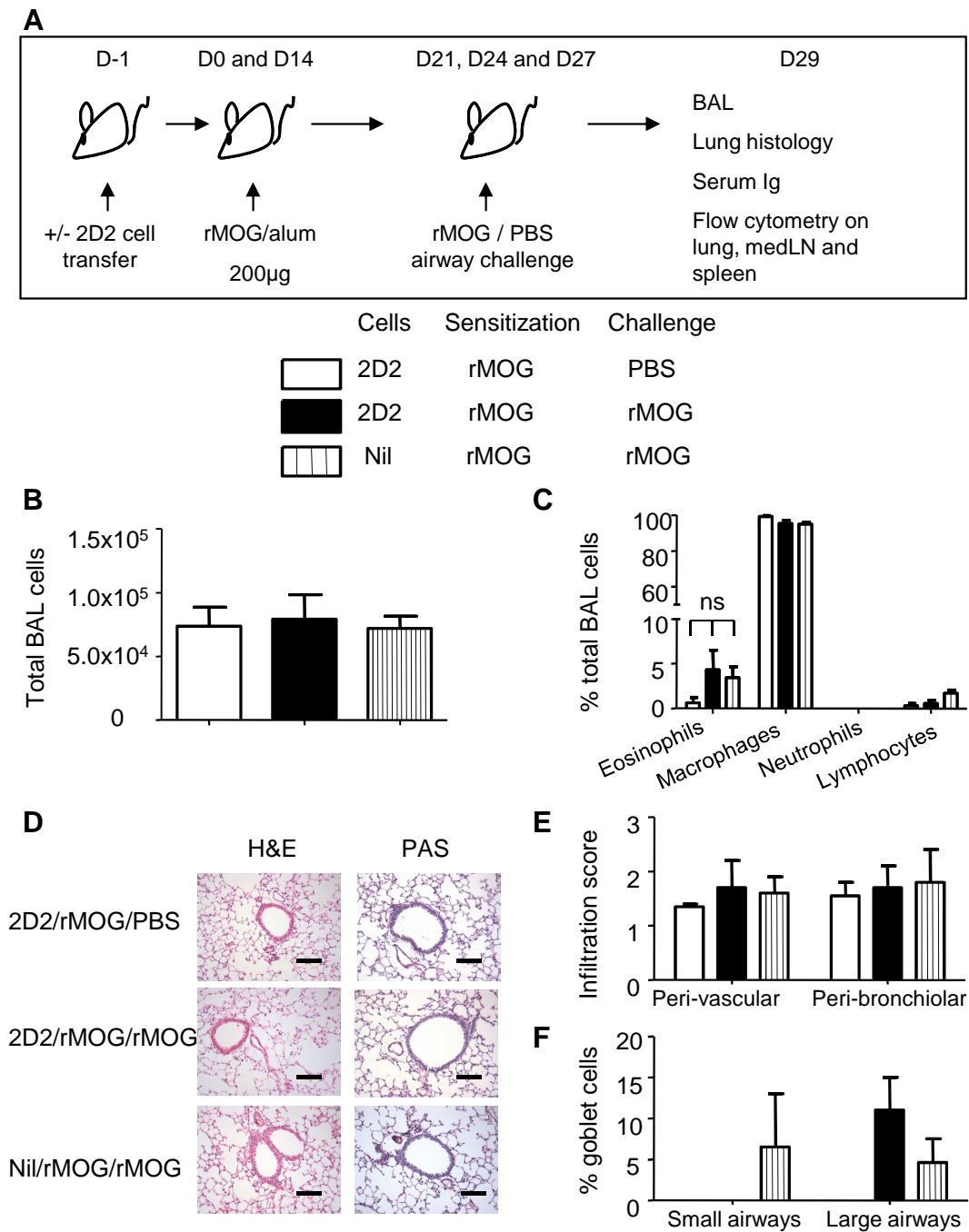


Figure 3.1 Sensitization and airway challenge with rMOG does not drive eosinophilia and lung inflammation in WT C57BL/6 mice.

Mice were sensitized with rMOG in alum and challenged with rMOG or PBS as a control. One of the rMOG challenged groups received traceable CD90.1+ naïve 2D2 CD4⁺ cells. (A) Experimental scheme. (B) Total numbers of BAL cells, $n=4$. (C) Percentages of cell types in BAL, $n=4$. (D) H&E and PAS staining of the lung tissue, $n=2$. (E) Infiltration scores and (F) percentages of goblet cells in the lung, $n=2$. Black line represents 100µm. Data are from one experiment. Similar data was obtained from experiment with 100 µg rMOG sensitization. Data are shown as mean \pm SEM. ns = not significant as determined by a one-way variance (ANOVA) using Tukey's multiple comparison test.

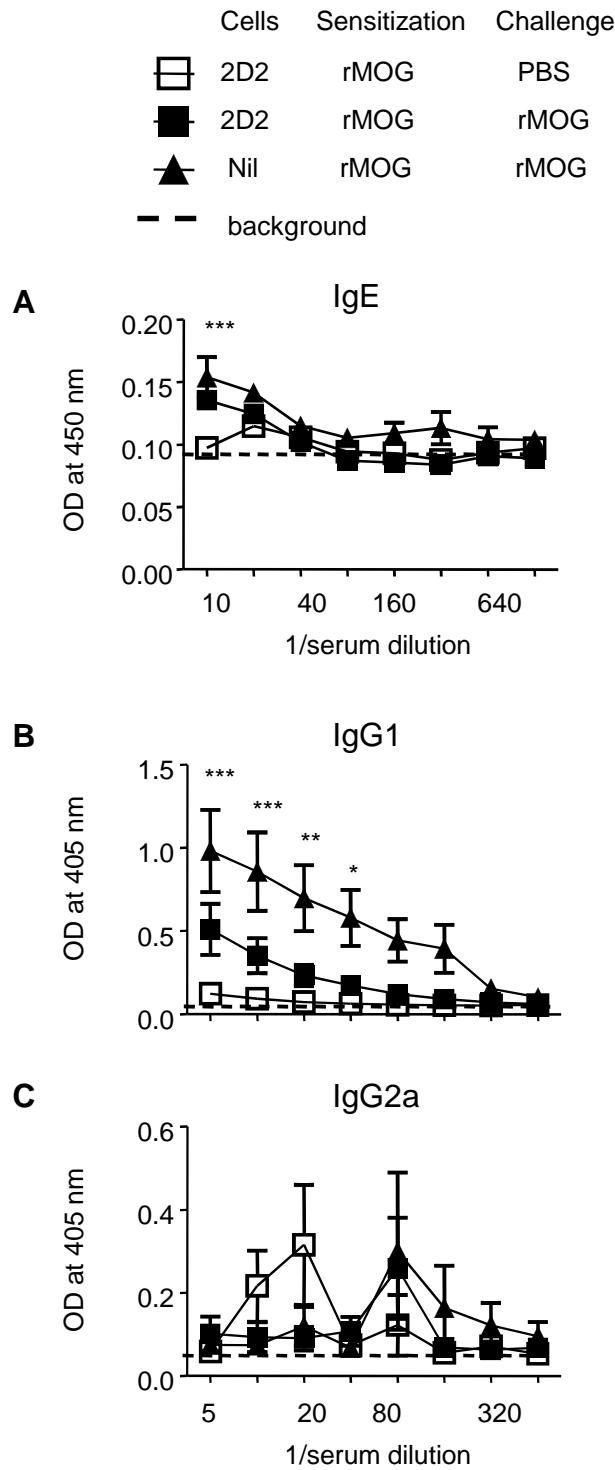


Figure 3.2 rMOG-specific IgE and IgG1 but not IgG2a are detected in serum from WT CD57BL/6 mice sensitized and challenged with rMOG.

Experimental approach is shown in Figure 3.1. (A) rMOG-specific IgE, (B) IgG1 and (C) IgG2a in serum. Data are from one experiment, $n=4$. Data are shown as mean \pm SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ between rMOG and PBS challenged groups as determined by a two-way variance (ANOVA) using Bonferroni's multiple comparison test.

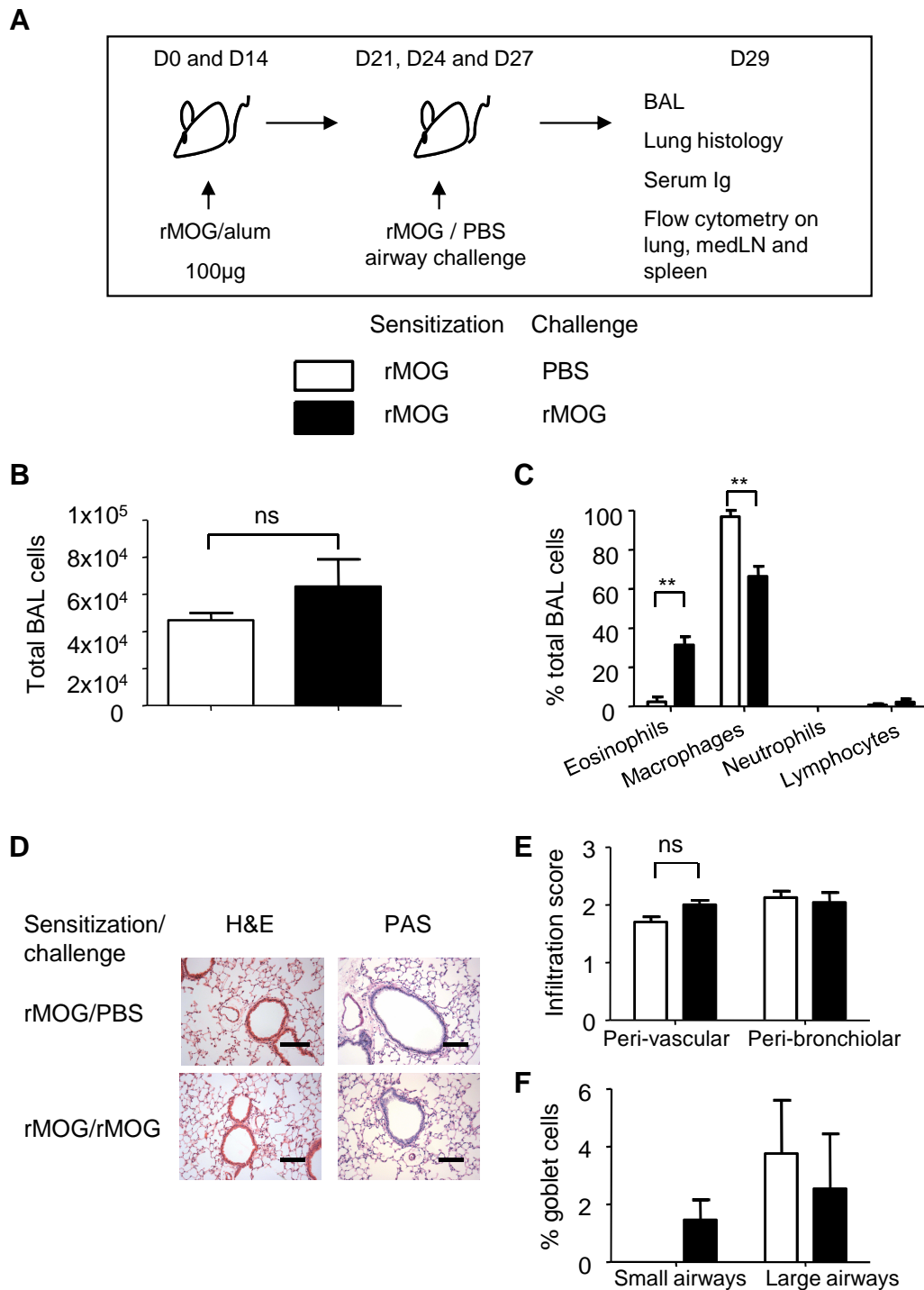


Figure 3.3 MOG-deficient mice develop lung eosinophilia but not lung inflammation.

MOG-deficient mice were sensitized with rMOG in alum and airway challenged with rMOG or PBS as a control. (A) Experimental scheme. (B) Total numbers of BAL cells. (C) Percentages of cell types in BAL. (D) H&E and PAS staining. (E) Infiltration scores and (F) percentages of goblet cells in the lung. Black line represents 100µm. Data are from one experiment, n=4-5. Data are shown as mean \pm SEM. **p<0.01, ns = not significant as determined by (B&C) an unpaired t-test and (E) Mann-Whitney U test.

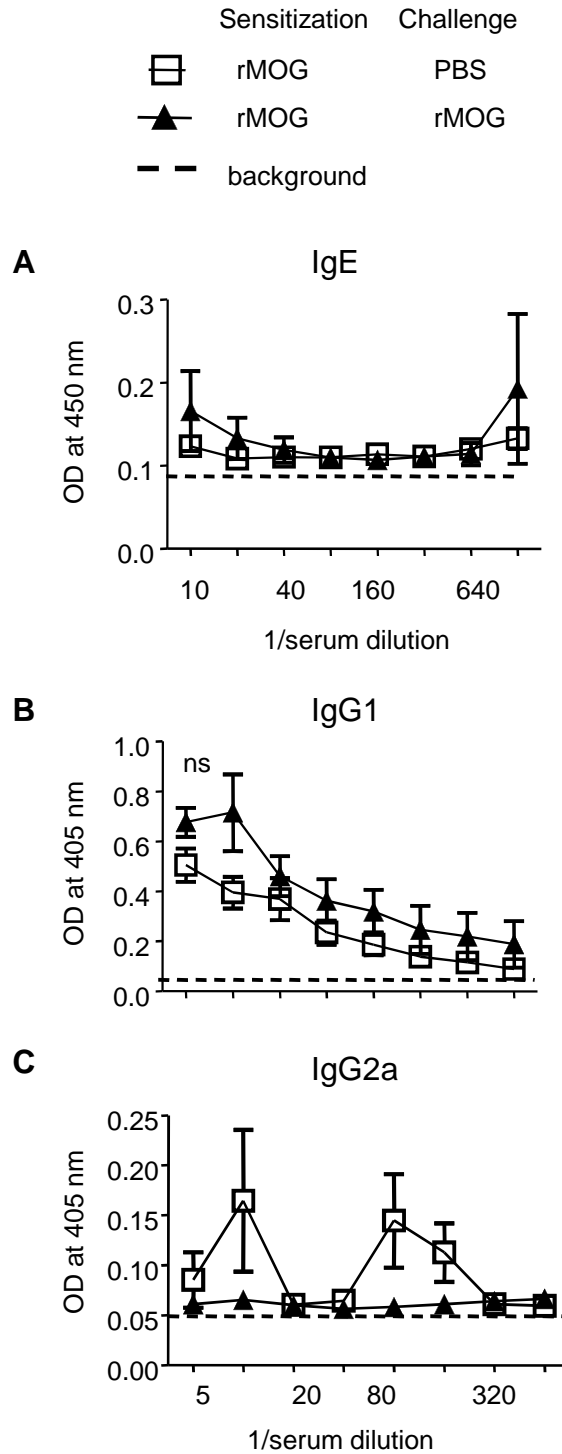


Figure 3.4 rMOG-specific IgE and IgG1 but not IgG2a are detected in serum from MOG-deficient mice sensitized with rMOG.

Experimental approach is shown in Figure 3.3. (A) rMOG-specific IgE, (B) IgG1 and (C) IgG2a in serum. Data are from one experiment, $n=4$. Data are shown as mean \pm SEM. ns=not significant between rMOG and PBS challenged groups as determined by an analysis of a two-way variance (ANOVA) using Bonferroni's multiple comparison test.

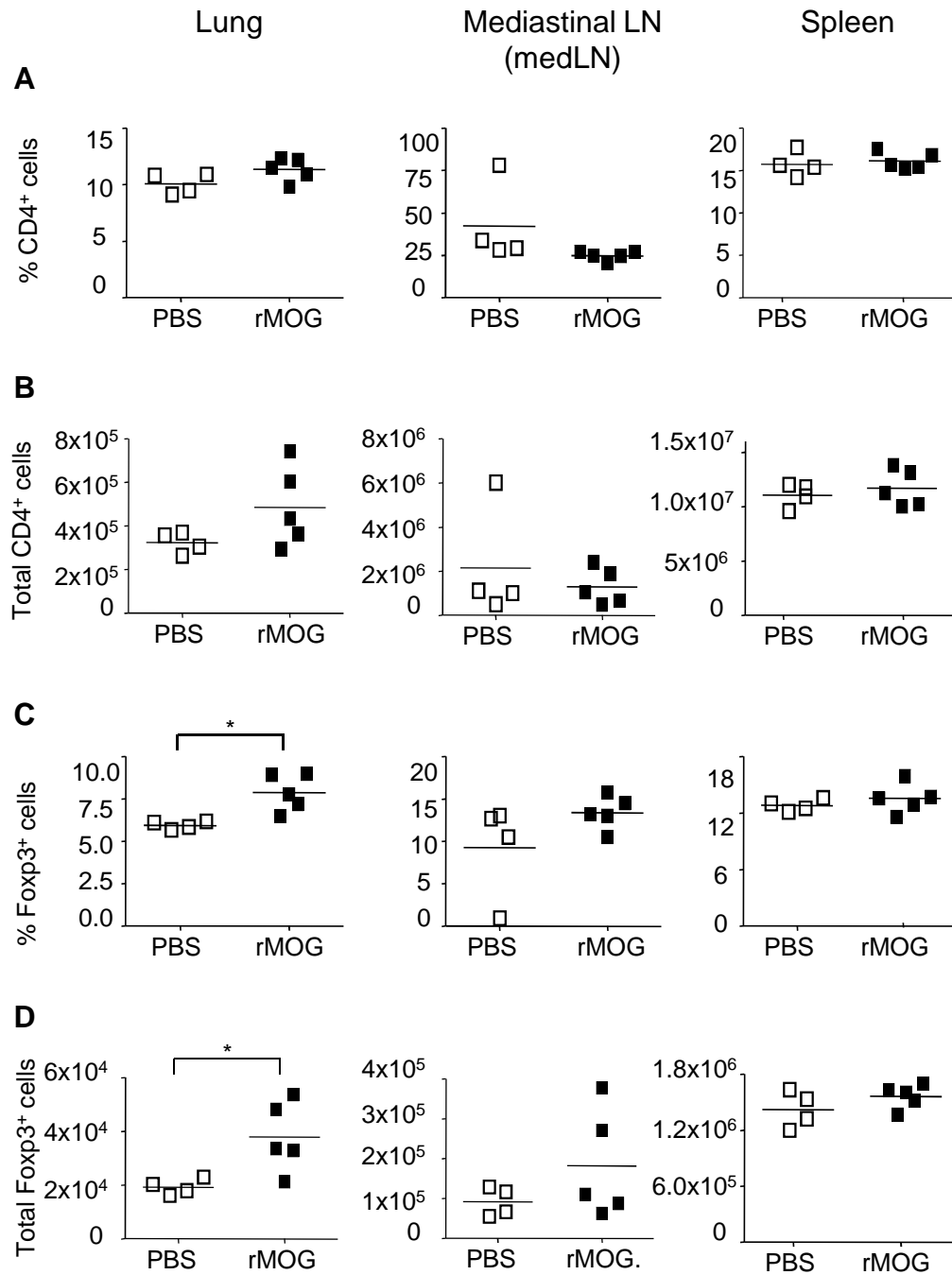


Figure 3.5 Antigen challenge in rMOG-sensitized MOG-deficient mice does not affect total CD4⁺ cells but significantly increases Foxp3 expression within the lung.

Experimental approach is shown in Figure 3.3. (A) Percentages of CD4⁺ cells of all cells. (B) Total numbers of CD4⁺ cells. (C) Percentages of Foxp3⁺ CD4⁺ cells of all CD4⁺ cells and (D) total numbers of Foxp3⁺ CD4⁺ cells. Data are from one experiment, n=4-5. *p<0.05 as determined by an unpaired t-test.

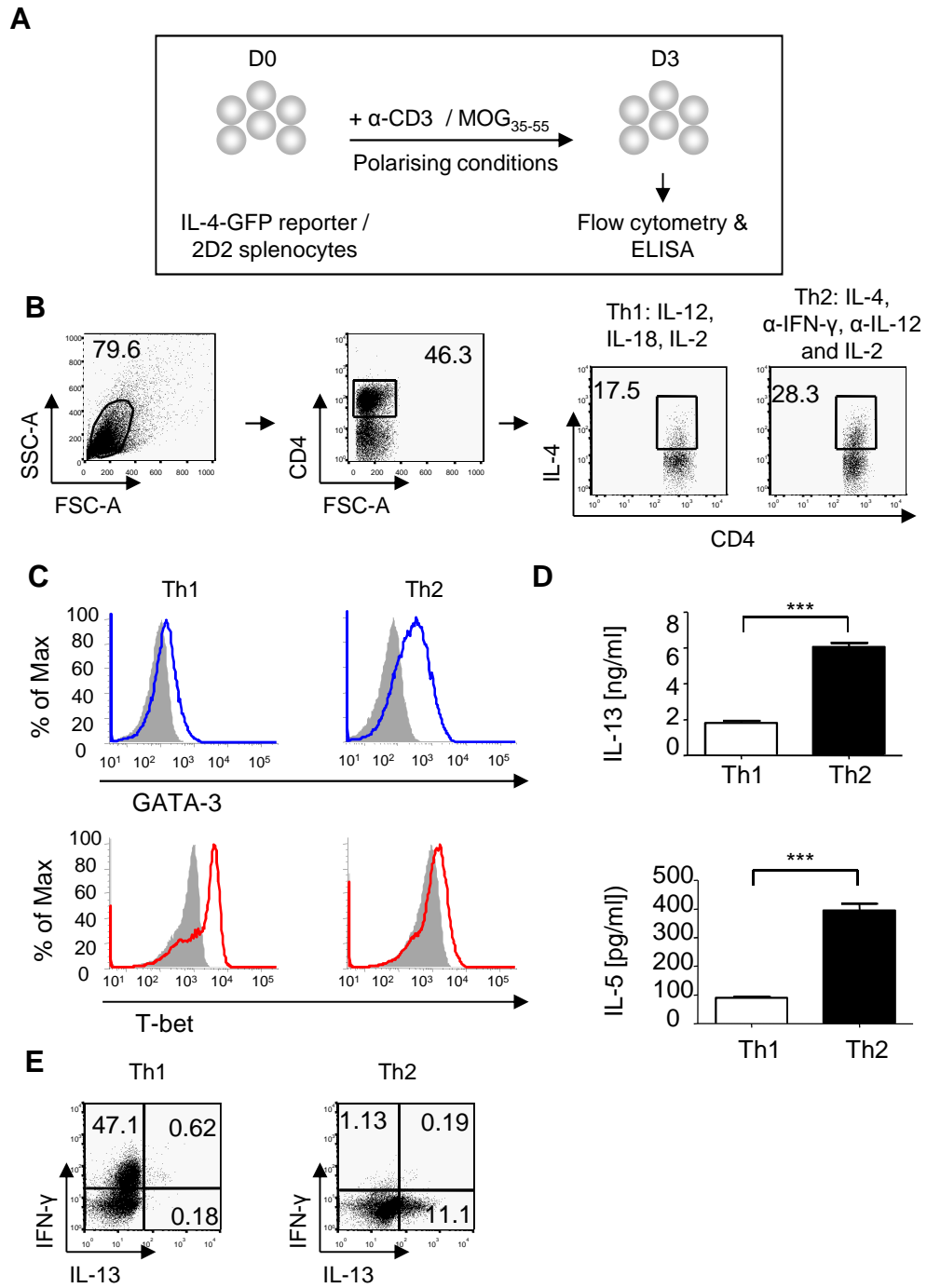


Figure 3.6 Optimisation of Th2 polarising conditions *in vitro*.

Splenocytes from IL-4-GFP reporter mouse or a 2D2 mouse were stimulated with α -CD3 or MOG₃₅₋₅₅, respectively, under different polarising conditions for 3 days. (A) Experimental scheme. (B) Flow cytometry plots showing gating strategy on CD4⁺ cells and IL-4 expression under different conditions. Numbers indicate percentages of gated cells within CD4⁺ cell population. (C) Expression levels of T-bet and GATA-3. Blue line marks GATA-3, red line marks T-bet and shaded area is an isotype control. (D) Cytokine production measured in cell culture supernatants by ELISA. (E) Intracellular cytokines produced by polarised 2D2 CD4⁺ cells. Data are representative of at least 4 experiments. Data are shown as mean \pm SEM. *** p <0.001 as determined by an unpaired t-test.

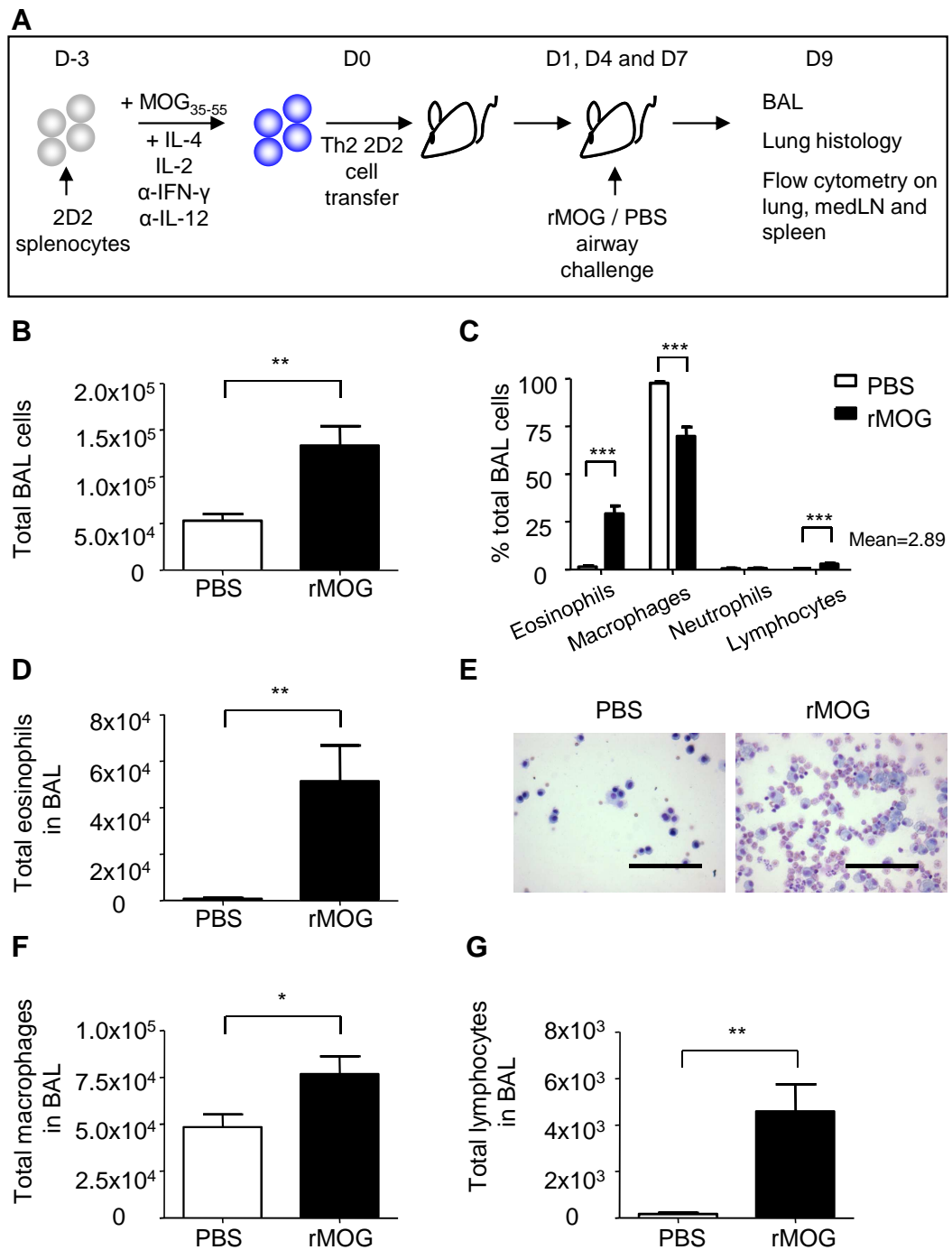


Figure 3.7 Adoptive transfer of Th2 polarised 2D2 CD4⁺ cells followed by rMOG challenge induces eosinophils in BAL consistent with AAI.

CD90.1⁺ 2D2 splenocytes were stimulated with MOG₃₅₋₅₅ under Th2 polarising conditions, sorted for CD4⁺ cells and transferred into WT mice followed by three intratracheal challenges with rMOG. (A) Experimental scheme. (B) Total cell numbers in BAL. (C) Percentages of cells in BAL. (D) Total numbers of eosinophils and (E) representative BAL cytopsin. (F) Total numbers of macrophages and (G) lymphocytes in BAL. Black line represents 100μm Data was pooled from 6 experiments, n=19-25. Data are shown as mean +/- SEM. *p<0.05, **p<0.01, ***p<0.001 as determined by an unpaired t-test.

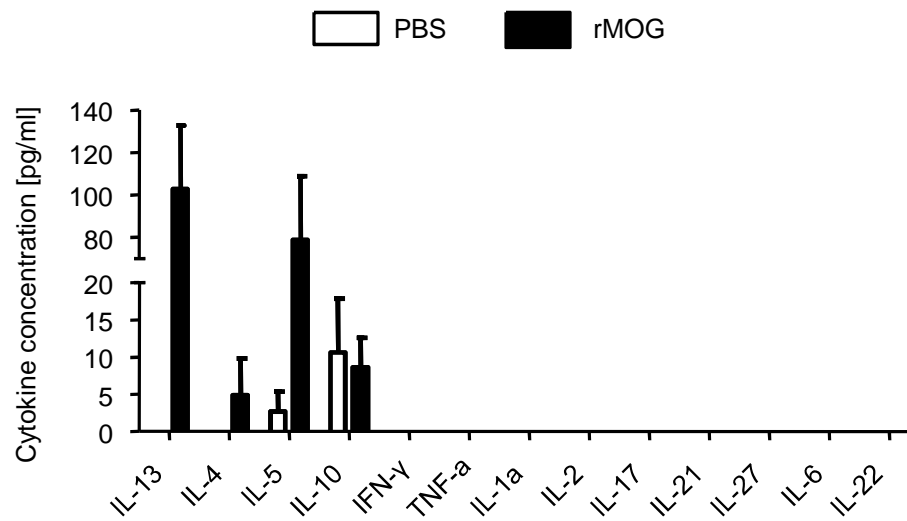
A

Figure 3.8 Th2-associated IL-13 and IL-5 are the predominant cytokines detectable in BAL fluid of rMOG-challenged mice.

Experimental approach is shown in Figure 3.7. Cytokines in BAL fluid were measured using 13-plex FlowCytomix. Data are from one experiment, n=3 Data are shown as mean \pm SEM.

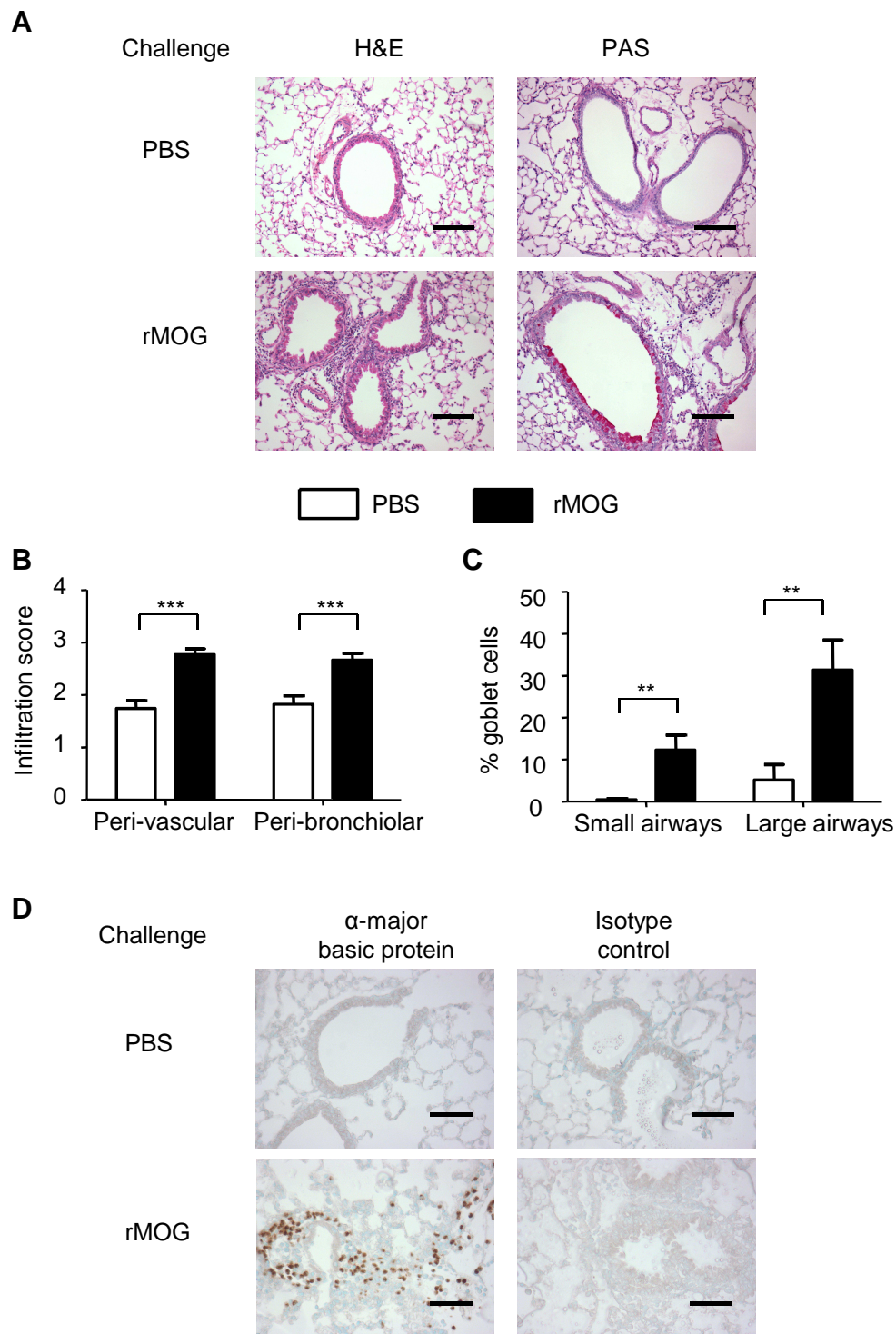
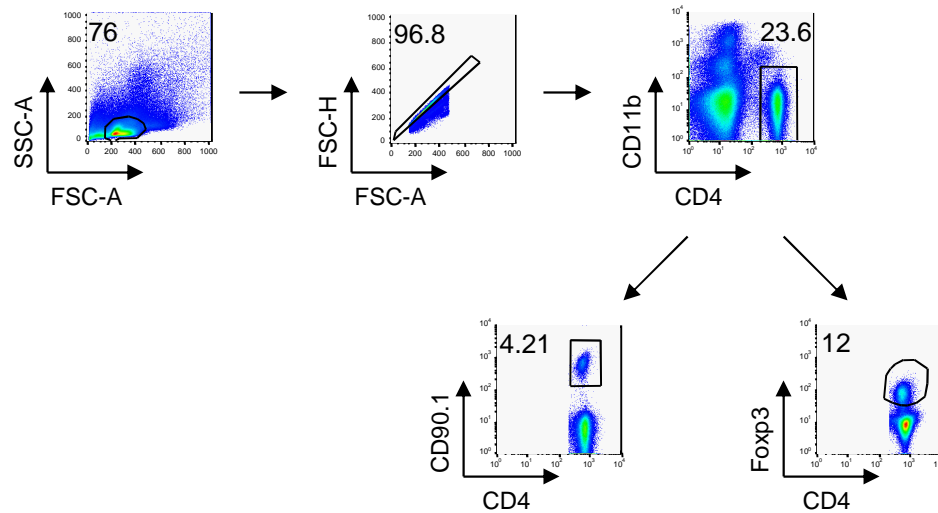


Figure 3.9 Th2 polarised 2D2 CD4⁺ T cells induce lung infiltration, goblet cell hyperplasia and lung eosinophilia upon airway challenge with rMOG.

Experimental approach is shown in Figure 3.7. (A) H&E and PAS staining (B) Infiltration scores and (C) percentages of goblet cells in the lung. (D) α -major basic protein staining for eosinophils. Black line represents 100 μ m. Data was pooled from 6 experiments, n=14-17. Data are shown as mean \pm SEM. * p <0.05, ** p <0.01, *** p <0.0001 as determined by (B) a Mann-Whitney U test and (C) an unpaired t-test.

A

Lung left lobe

medLN

Spleen

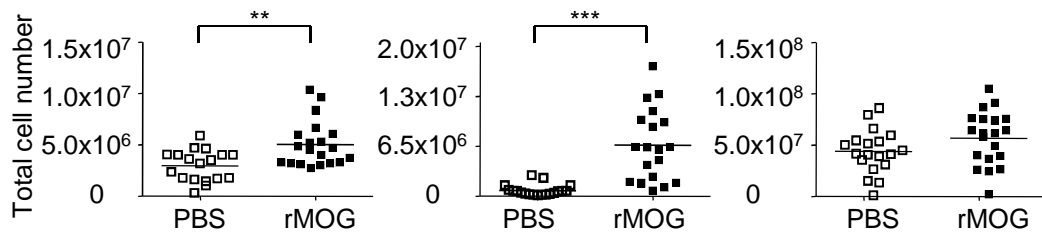
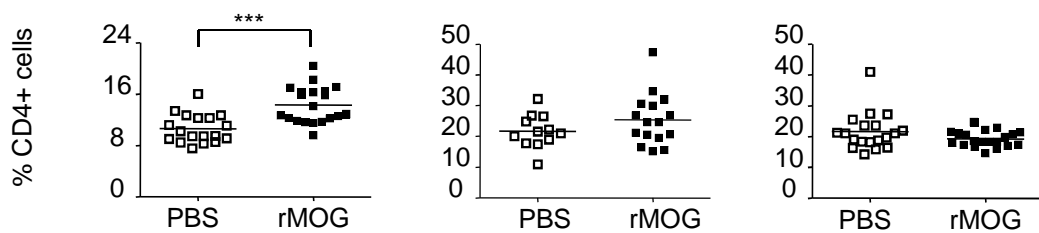
B**C**

Figure 3.10 Increased total cell numbers in the lung and medLN, and an increased percentage of total CD4⁺ cells within the lung following transfer of Th2 2D2 cells and rMOG challenge.

Experimental approach is shown in Figure 3.7. (A) Representative flow cytometry plots showing the gating strategy for CD4⁺, Foxp3⁺ and Th2 2D2 (CD90.1⁺) cell populations. (B) Total cell numbers and (C) percentages (%) of CD4⁺ T cells of all cells. Data was pooled from 5 experiments, n=12-20. **p<0.01, ***p<0.001 as determined by an unpaired t-test.

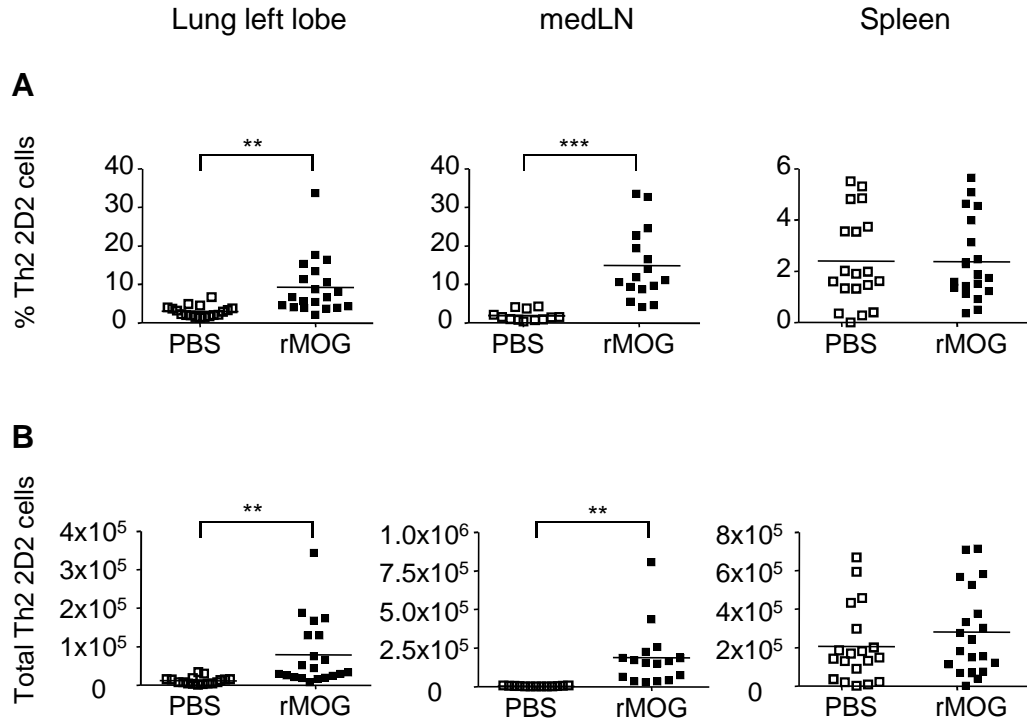


Figure 3.11 Percentages and total numbers of Th2 2D2 cells increase upon rMOG challenge specifically within the lung and medLN.

Experimental approach is shown in Figure 3.7 and the gating strategy in Figure 3.10. (A) Percentages of Th2 2D2 cells of all CD4⁺ cells and (B) total cell numbers of Th2 2D2 cells. Data was pooled from 5 experiments, n=12-20. **p<0.01, ***p<0.001 as determined by an unpaired t-test.

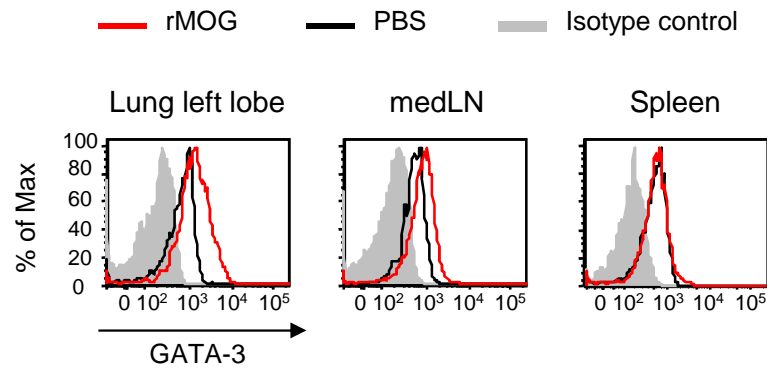
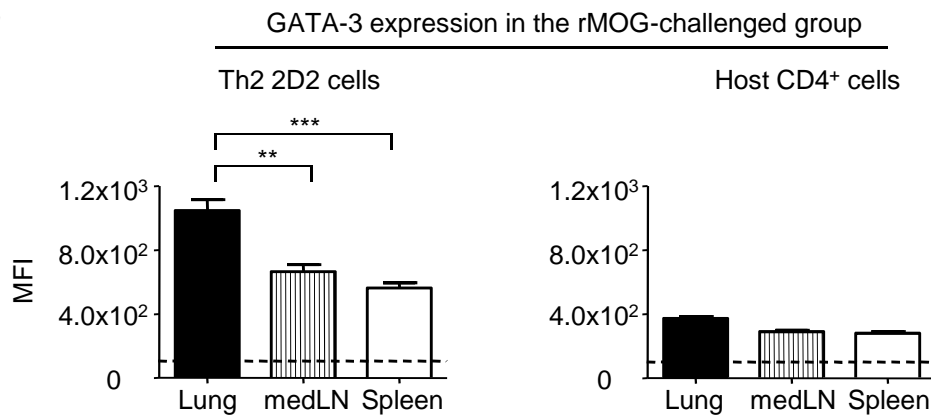
A**B**

Figure 3.12 Transferred Th2 2D2 cells retain the highest expression of GATA-3 in the rMOG-challenged lung.

Experimental approach is shown in Figure 3.7 and the gating strategy in Figure 3.10. (A) Representative histograms of GATA-3 expression in Th2 2D2 cells in rMOG- and PBS-challenged groups. (B) Mean fluorescence intensity (MFI) of GATA-3 expression in Th2 2D2 cells and host CD4⁺ cells in the rMOG-challenged group. Dotted lines represent isotype control. Data are representative of 3 experiments, n=4. Data are shown as mean \pm SEM. **p < 0.01, ***p < 0.001 as determined by a one-way variance (ANOVA) using Tukey's multiple comparison test.

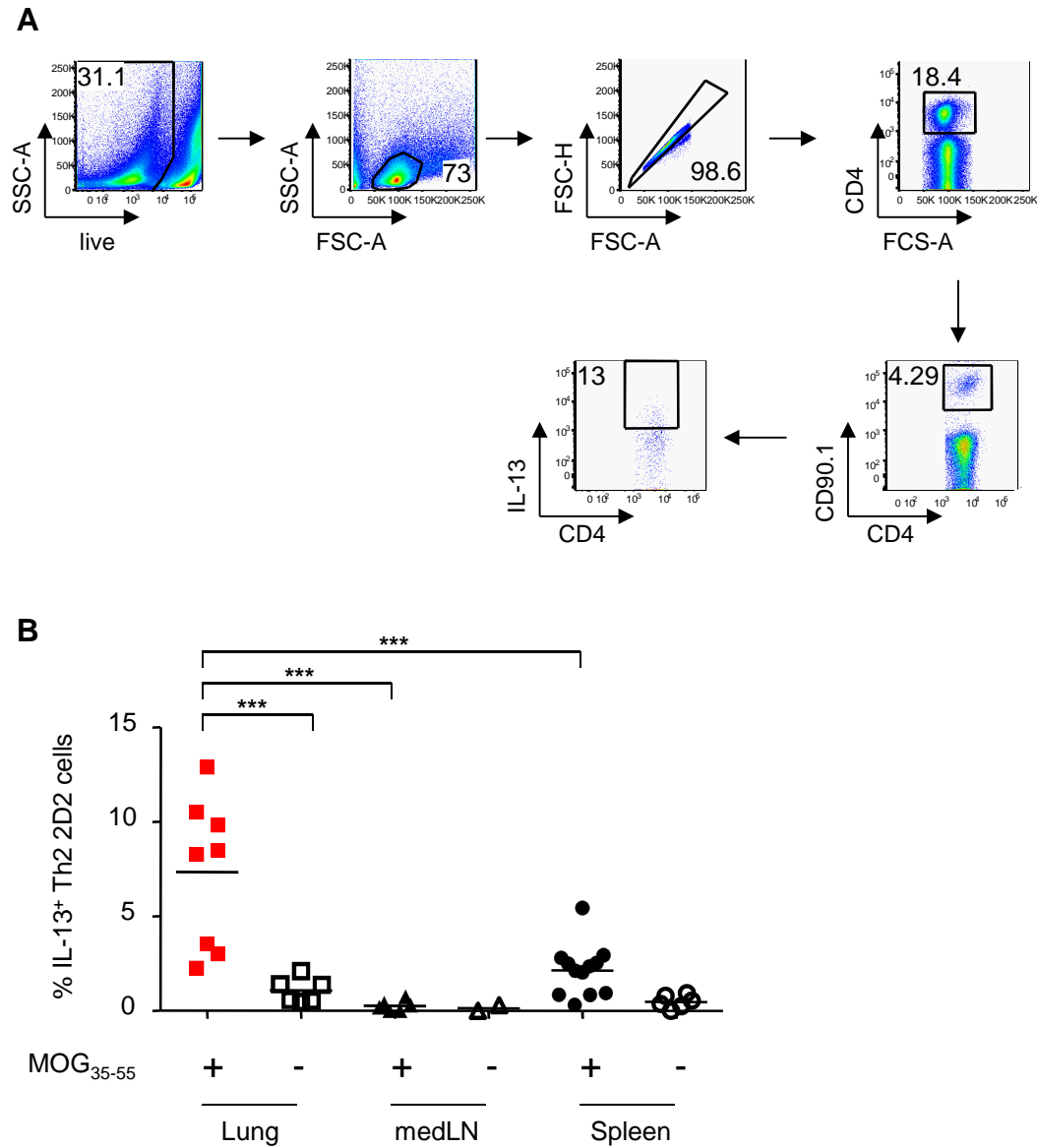


Figure 3.13 Transferred Th2 2D2 cells from the challenged lung retain ability to produce Th2-associated IL-13.

Experimental approach is shown in Figure 3.7. On day 9 cells isolated from the lung, medLN and spleen were cultured with (+) or without (-) MOG₃₅₋₅₅ overnight and effector cytokine production was determined by ICS. A) Representative flow cytometry plots showing the gating strategy for Th2 2D2 (CD90.1⁺) and IL-13⁺ Th2 2D2 cell populations. (B) Percentages of IL-13⁺ Th2 2D2 cells. Each data point from the lung and medLN represents 2-3 pooled mice. Data are pooled from 4 experiments for lung and spleen, and from 2 experiments for medLN. ***p<0.001 as determined by a one-way variance (ANOVA) using Tukey's multiple comparison test.

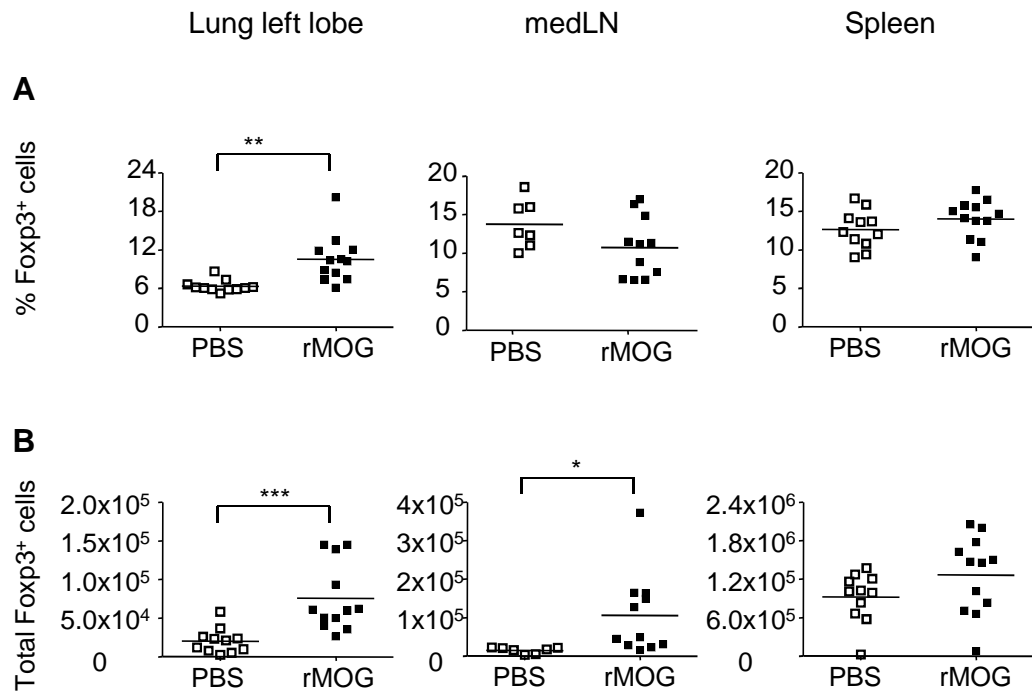


Figure 3.14 Percentages and absolute cell numbers of total Foxp3⁺ CD4⁺ cells increase upon rMOG challenge specifically within the lung.

Experimental approach is shown in Figure 3.7 and the gating strategy in Figure 3.10. (A) Percentages of Foxp3⁺ CD4⁺ cells of all CD4⁺ cells and (B) total cell numbers of Foxp3⁺ CD4⁺ cells. Data are pooled from 3 experiments, n=7-12. *p<0.5, **p<0.01, ***p<0.001 as determined by an unpaired t-test.

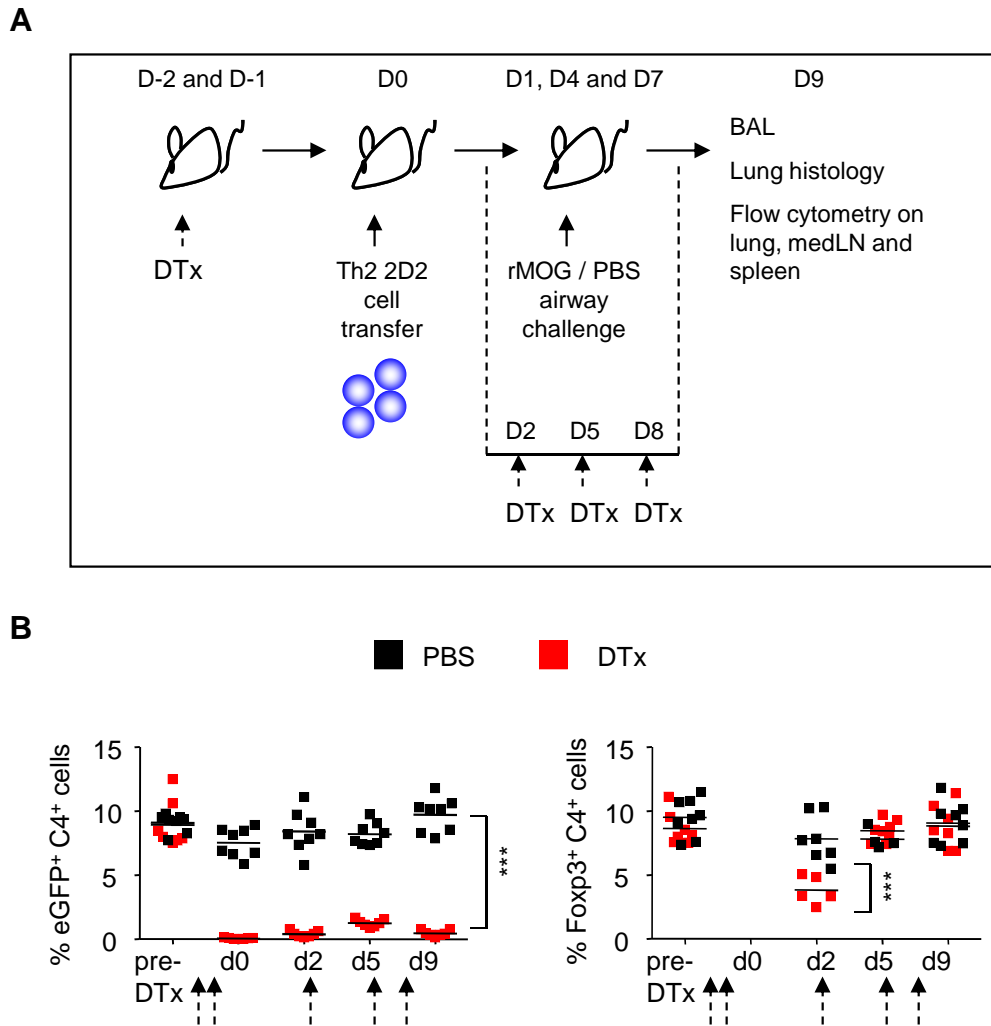


Figure 3.15 Depletion of Foxp3⁺ cells following diphtheria toxin (DTx) treatment in Foxp3.LuciDTR4 mice during and throughout the induction of AAI.

Foxp3.LuciDTR-4 mice were given DTx or PBS on two consecutive days before transferring traceable CD90.1⁺ Th2 2D2 cells and received further 3 doses on days 2, 5 and 8. Blood was sampled prior to DTx treatment and then every 2 to 4 days to determine depletion of Foxp3⁺ cells by either eGFP expression or ICS for the expression of Foxp3 protein. (A) Experimental scheme. (B) Percentages of eGFP⁺ CD4⁺ cells and Foxp3⁺ CD4⁺ cells of total CD4⁺ cell population pre- and post-DTx treatment. Due to insufficient cell numbers there is no data for the ICS expression of Foxp3 on day 0. Dashed arrows mark DTx injections. Data are from one experiment, n=5-8. ***p<0.001 as determined by a two-way variance (ANOVA) using Bonferroni's multiple comparison test.

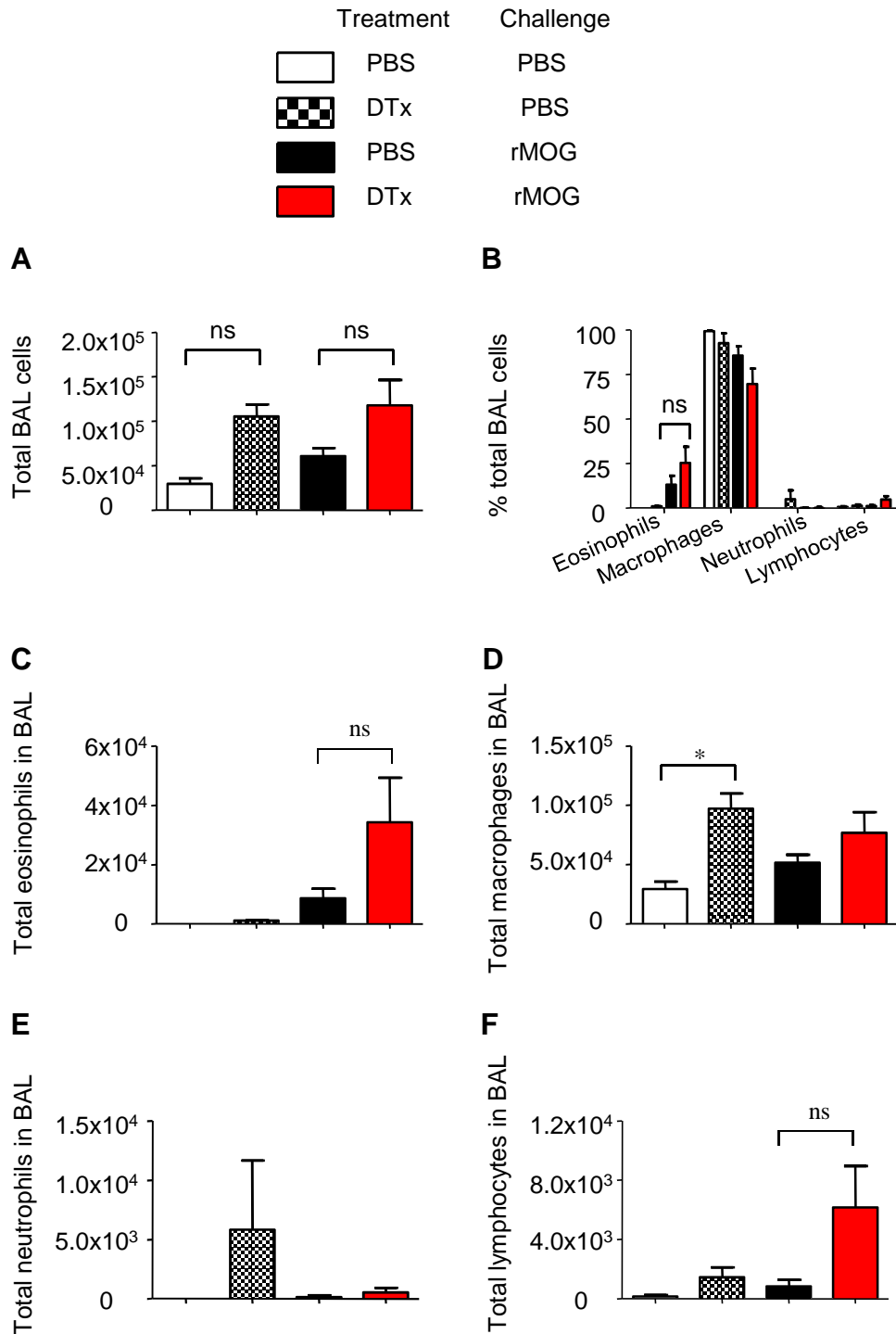
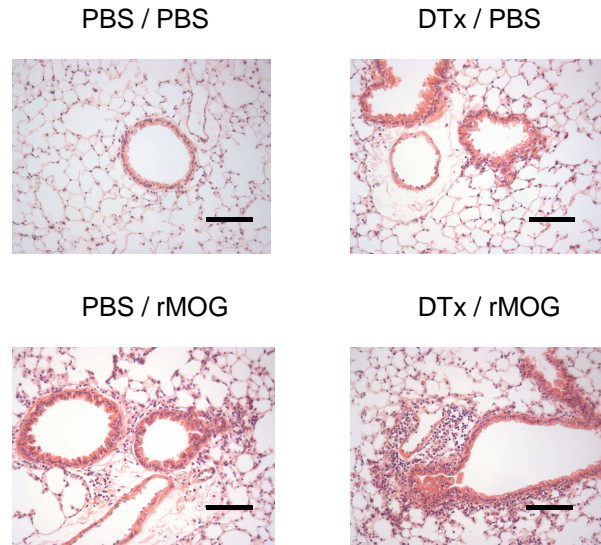










Figure 3.16 Depletion of Foxp3⁺ cells prior to and during induction of AAI does not significantly affect the percentage and number of BAL eosinophils.

Experimental approach is shown in Figure 3.15. (A) Total numbers of cells and (B) percentages of cells in BAL. (C) Total cell numbers of eosinophils, (D) macrophages, (E) neutrophils and (F) lymphocytes in BAL. Data are from one experiment, n=3-4. Data are shown as mean \pm SEM. ns = not significant, *p<0.05 as determined by a one-way variance (ANOVA) using Tukey's multiple comparison test.

A

Treatment		Challenge	
	PBS		PBS
	DTx		PBS
	PBS		rMOG
	DTx		rMOG

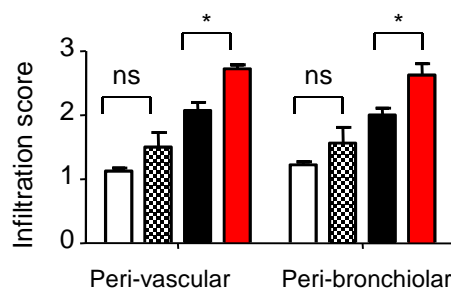
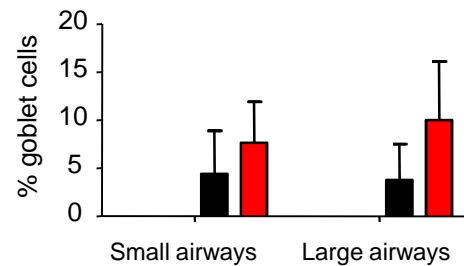
B**C**

Figure 3.17 Depletion of Foxp3⁺ cells resulted in the increased lung infiltration but did not affect percentages of goblet cells.

Experimental approach is shown in Figure 3.15. (A) H&E staining. (B) Infiltration scores and (C) percentages of goblet cells in the lung. Black line represents 100μm. Data are from one experiment, n=3-4. Data are shown as mean +/- SEM. *p<0.05 as determined by a Mann-Whitney U test.

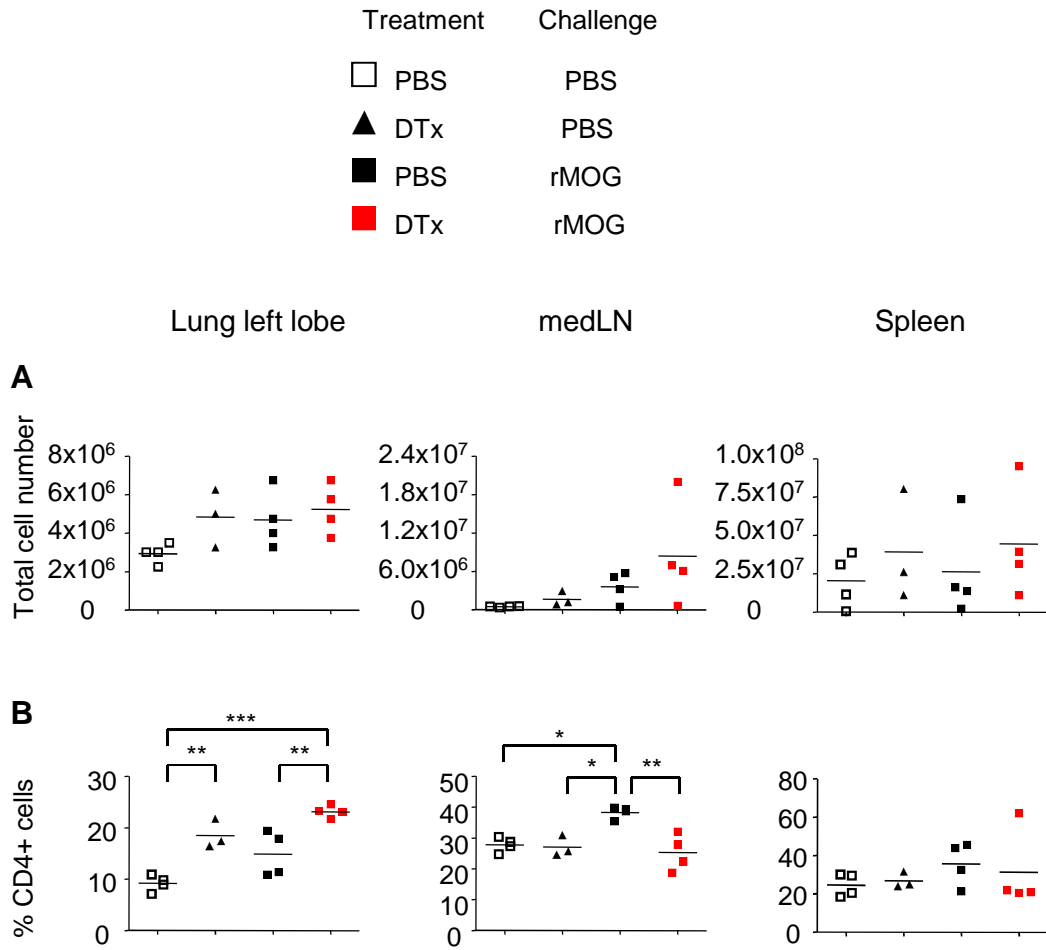


Figure 3.18 Depletion of Foxp3⁺ cells prior to and during AAI induction resulted in the increased percentage of total CD4⁺ cells within the lung.

Experimental approach is shown in Figure 3.15. (A) Total cell numbers. (B) Percentages of CD4⁺ cells of all cells. Data are from one experiment, n=3-4. *p<0.05, **p<0.01, ***p<0.001 as determined by a one-way variance (ANOVA) using Tukey's multiple comparison test.

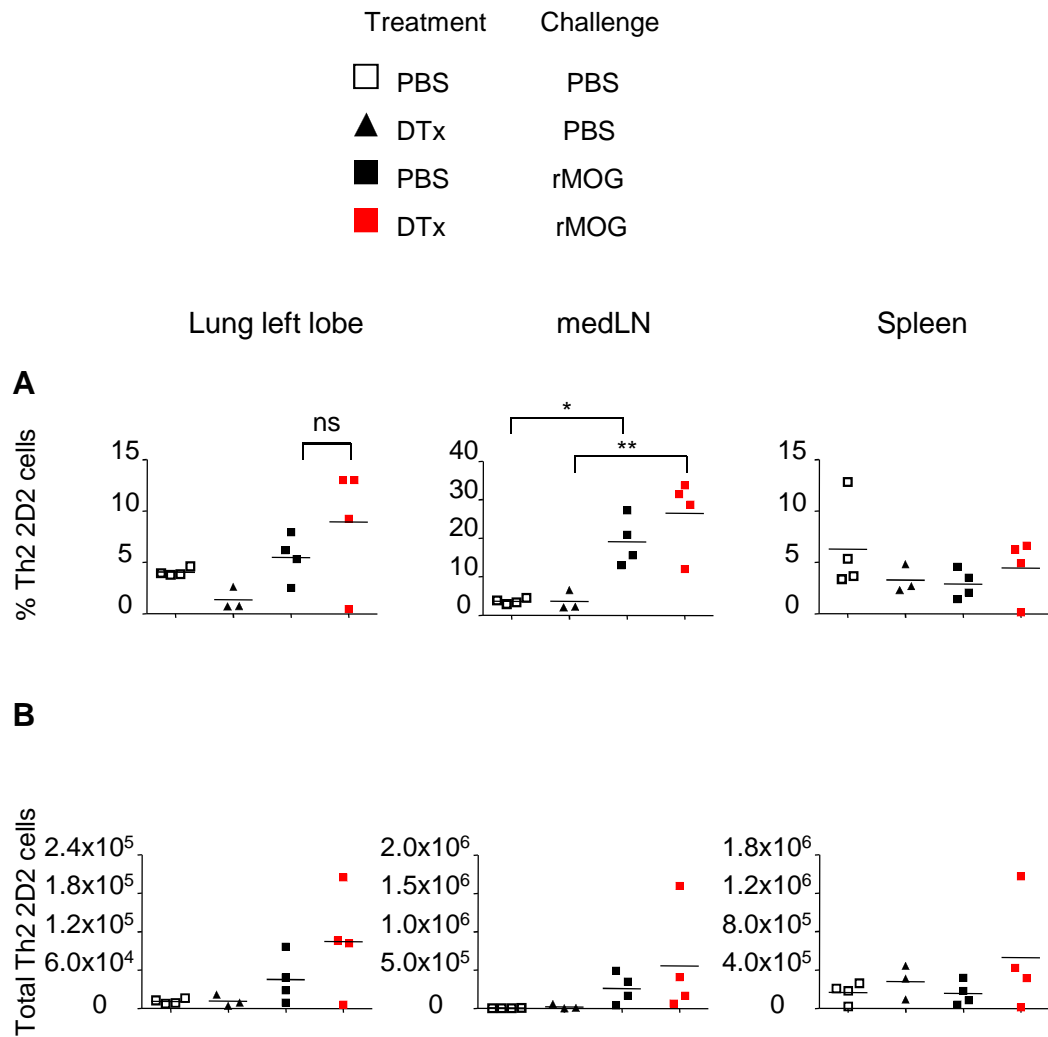


Figure 3.19 Depletion of Foxp3^+ cells prior to and during AAI induction did not affect percentages and total numbers of Th2 2D2 cells within the lung and medLN.

Experimental approach is shown in Figure 3.15. (A) Percentages of Th2 2D2 cells of all CD4^+ cells and (B) total cell numbers of Th2 2D2 cells in the left lung lobe, medLN and spleen. Data are from one experiment, $n=3-4$. ns = not significant, $*p<0.05$, $**p<0.01$ as determined by a one-way variance (ANOVA) using Tukey's multiple comparison test.

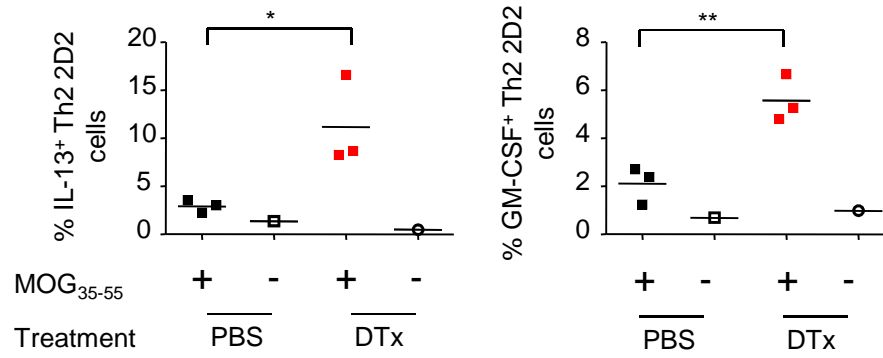


Figure 3.20 Increased frequency of IL-13⁺ and GM-CSF⁺ Th2 2D2 cells from the lung of mice depleted of Foxp3⁺ cells.

Experimental approach is shown in Figure 3.15. On day 9 cells isolated from the lungs of rMOG-challenged mice were cultured with (+) or without (-) MOG₃₅₋₅₅ overnight and effector cytokine production was determined by ICS. Cells from unstimulated controls were pooled. Data are from one experiment. * $p < 0.05$, ** $p < 0.01$ as determined by an unpaired t-test.

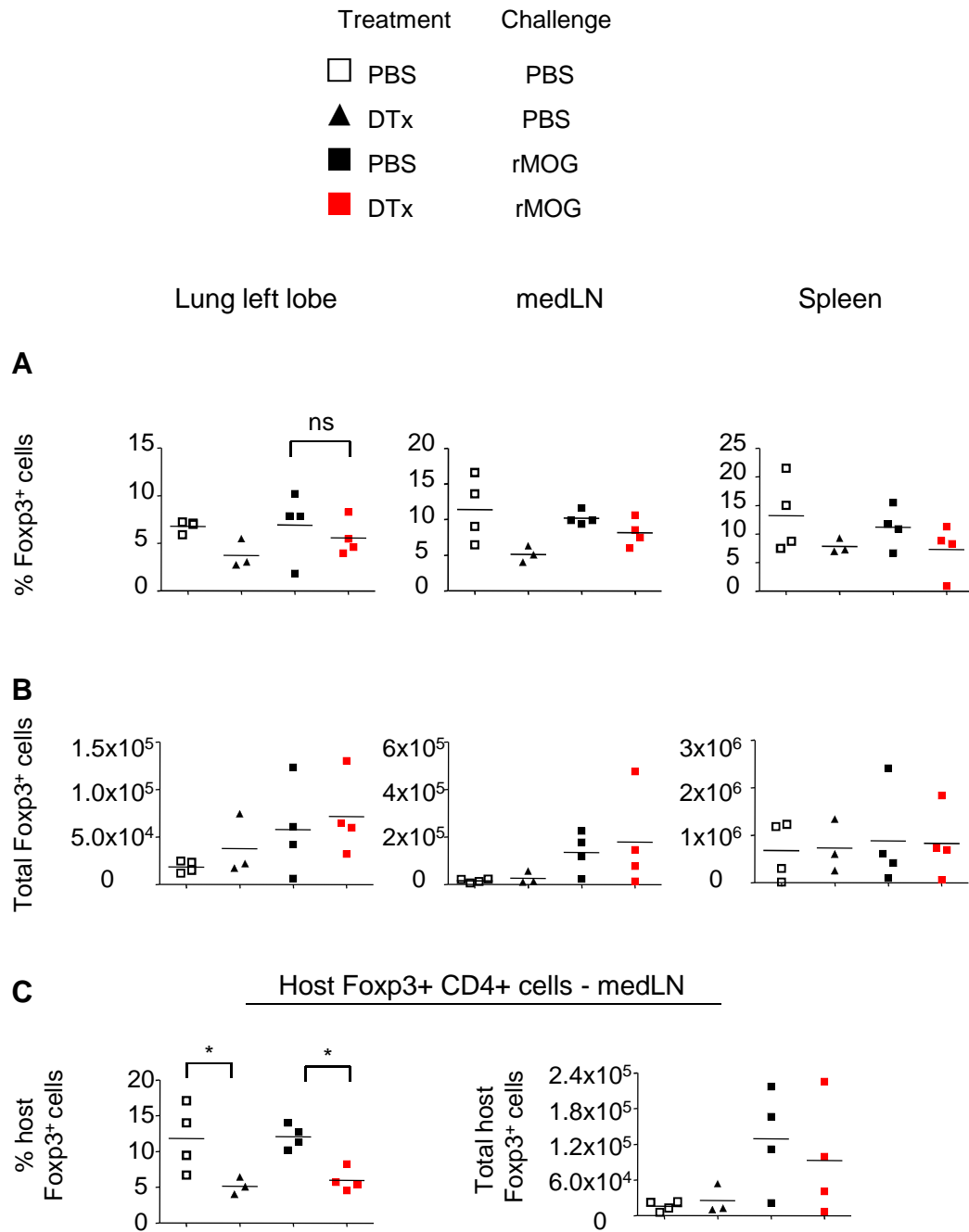


Figure 3.21 Administration of DTx prior to and during AAI induction did not affect the percentage and absolute numbers of total Foxp3⁺ CD4⁺ cells but decreased percentage of host Foxp3⁺ cells in the medLN.

Experimental approach is shown in Figure 3.15. Intracellular staining was used to detect Foxp3 expression. (A) Percentages of total Foxp3⁺ CD4⁺ cells of all CD4⁺ cells and (B) total numbers of Foxp3⁺ CD4⁺ cells in the left lung lobe, medLN and spleen. (C) Percentage (of host CD4⁺ cells) and total number of host Foxp3⁺ cells in medLN. Data are from one experiment, n=3-4. ns = not significant, *p<0.05 as determined by a one-way variance (ANOVA) using Tukey's multiple comparison test.

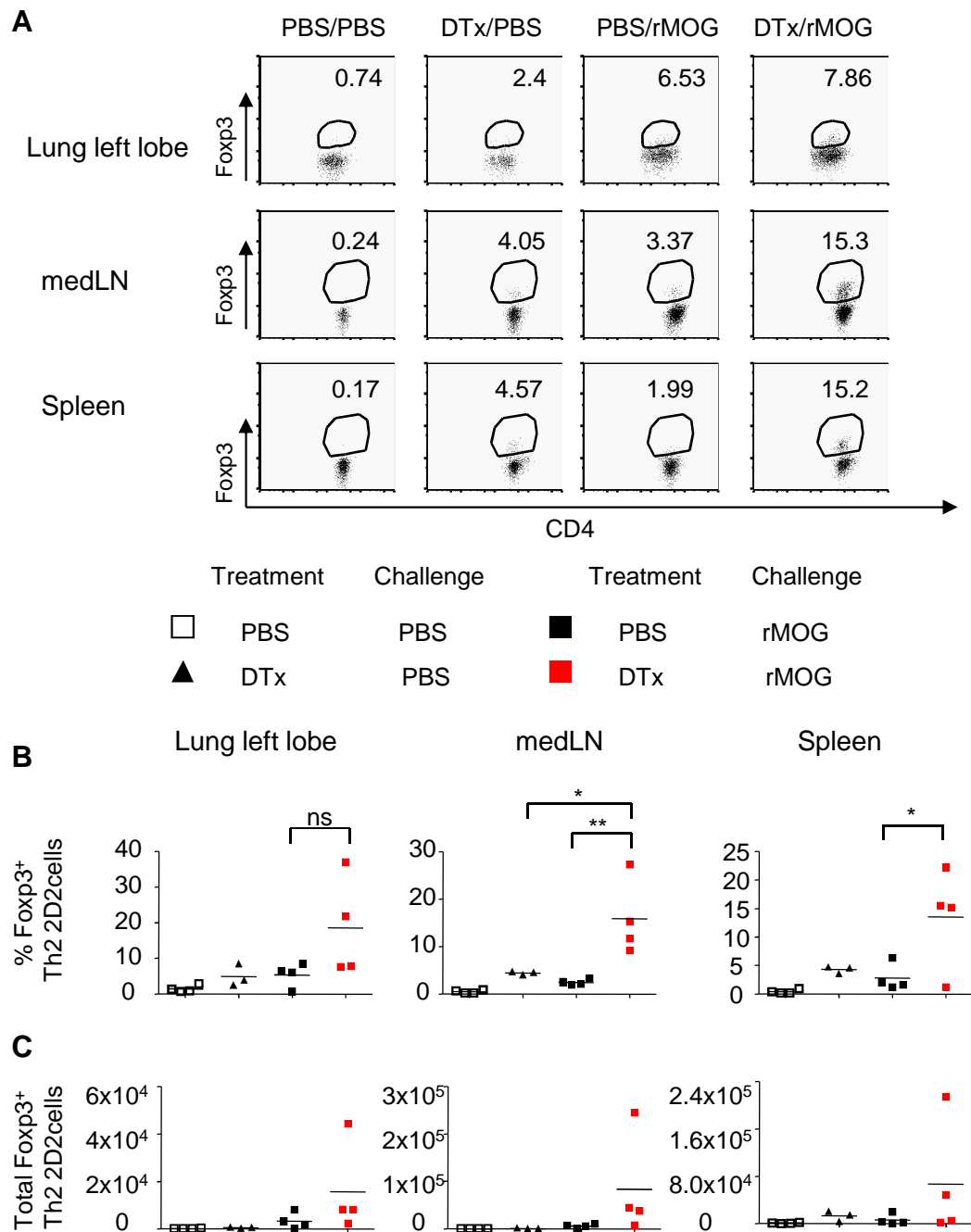


Figure 3.22 Depletion of host Foxp3⁺ cells resulted in the increased frequency of Foxp3⁺ Th2 2D2 cells in the medLN and spleen.

Experimental approach is shown in Figure 3.15. Intracellular staining was used to detect Foxp3 expression. (A) Representative flow cytometry plots showing percentages of Foxp3⁺ Th2 2D2 cells of total Th2 2D2 cells. (B) Percentages of Foxp3⁺ Th2 2D2 cells of total Th2 2D2 cells and (C) total numbers of Foxp3⁺ Th2 2D2 cells in the left lung lobe, medLN and spleen. Data are from one experiment, n=3-4. ns = not significant, *p<0.05, **p<0.01 as determined by a one-way variance (ANOVA) using Tukey's multiple comparison test.

3.3. Discussion

3.3.1. Why does rMOG sensitization do not induce lung inflammation?

In this project the sensitization of C57BL/6 mice with rMOG and subsequent airway challenges were not found to induce eosinophilia and lung inflammation characteristic of AAI. Detection of rMOG-specific IgE and IgG1, associated with Th2 responses, suggested that mice were successfully sensitized. However, this was not sufficient to promote the inflammatory response in the lung. A possible explanation for this observation could be immune tolerance towards MOG as it is a self-antigen. As described in section 1.3.2, a number of mechanisms exist to prevent uncontrollable T cell responses towards self-antigens which can otherwise lead to autoimmunity (Ryan et al., 2007). Nevertheless, it is known that rMOG can effectively induce Th1/Th17-mediated autoimmune CNS inflammation as seen in EAE which is induced by immunization of WT mice with rMOG in CFA (Fillatreau et al., 2002). Alum used in a sensitization is a less strong adjuvant than CFA and although it was shown to promote production of IgE and IgG1, the Th2-associated antibody response induced by rMOG in alum might not be sufficiently robust for an induction of AAI.

In order to verify whether a degree of self-tolerance to MOG was implicated in the failure to induce AAI, the lung inflammatory reaction was investigated in MOG-deficient mice, in which rMOG can be considered a foreign antigen. Despite ~35% of eosinophils in BAL no lung infiltration or goblet cell hyperplasia was evident in these mice. The sensitization was confirmed by the detection of rMOG-specific IgE and IgG1 in serum, although the levels of IgE were low and did not increase upon rMOG challenge. This suggests that possible self-tolerance mechanisms to MOG are not implicated in the lack of lung inflammation following AAI protocol.

An explanation for the lack of allergic inflammatory reaction in the lung observed in both WT and MOG-deficient mice could be poor allergenic properties of rMOG. As already described in section 1.8.1, it is not known what constitutes an allergen although enzymatic activity has been implicated in allergenicity of some peptides

such as HDM-derived Der p1 (Schulz et al., 1998). It has been determined that an allergen such as OVA₃₂₃₋₃₃₉ has to have at least two allergenic determinants that bind to IgE on mast cells and cross link them to cause mast cell degranulation (Honma et al., 1996). It is possible that rMOG which is not a natural allergen may not have the properties that would make it a true allergen even though it can be considered a foreign antigen in MOG-deficient mice. A contributing factor could be the strain of mice used in this project. Both WT and MOG-deficient mice used in all experiments described in this thesis are on the C57BL/6 background and it has been demonstrated by multiple studies that C57BL/6 mice are less susceptible to developing allergic airway inflammation and as they do not generally display detectable AHR most induction protocols use BALB/c mice (Gueders et al., 2009, Conrad et al., 2009, Sahu et al., 2010).

3.3.2. How does rMOG-induced AAI mediated by Th2 2D2 cells compare to other models of AAI?

The majority of AAI induction protocols use antigen sensitization to initiate allergic responses. However, in this project although sensitization with rMOG was found to induce IgE and IgG1 no inflammatory response in the lung was observed. That is why a different approach was taken - the adoptive transfer of Th2 polarised MOG₃₅₋₅₅-reactive (2D2) CD4⁺ cells. The use of antigen-reactive Th2 cells to induce AAI in response to airway challenge with the antigen has been demonstrated in several studies. Cohn et al. used Th2 polarised OVA₃₂₃₋₃₃₉-reactive CD4⁺ cells followed by 7 days of daily exposure to aerosolized OVA in BALB/c mice (Cohn et al., 1997). In this project a different method of antigen challenge was chosen as it was previously determined that intratracheal challenge results in more direct and even distribution of the antigen within the lung versus intranasal challenge (Sarah Howie, personal communication). Although the experiments described in this chapter involved a similar Th2 polarisation protocol and the same number of transferred antigen-reactive Th2 cells as by Cohn et al., most readouts of disease were strikingly less severe. As no infiltration scores or total cell numbers in the lung were given in that study it is difficult to make a direct comparison in terms of lung pathology. However, total cell numbers in BAL and eosinophil counts were approximately 20-

fold lower in rMOG-induced AAI. Similarly, goblet cell hyperplasia reached ~65% in the Cohn study compared to ~12% to 32% observed here. The most obvious explanations for these observations would be a different airway challenge protocol and a different strain of mouse used.

Interestingly, when the percentages of antigen-reactive Th2 cells were compared, OVA₃₂₃₋₃₃₉-reactive Th2 cells comprised ~8% of all CD4⁺ cells in the medLN and 75% in the lung in the Cohn study versus ~15% of MOG₃₅₋₅₅-reactive Th2 cells in the medLN and only 9% in the lung in rMOG-induced AAI. Considering the higher percentage of Th2 2D2 cells within medLN and much lower in the lung compared to OVA₃₂₃₋₃₃₉-reactive Th2 cells, it is possible that these cells differ in their ability to migrate into the lung. The Cohn study demonstrated that IL-4 production by Th2 polarised OVA₃₂₃₋₃₃₉-reactive CD4⁺ cells was critical to their recruitment into the lung (Cohn et al., 1997). Th2 2D2 cells were shown to produce IL-4 upon re-stimulation *in vitro* (data not shown). However, low IL-4 concentrations detected in BAL fluid of rMOG-challenged mice could suggest that there is insufficient IL-4 produced by Th2 2D2 cells *in vivo* for their more efficient recruitment into the lung. The lower percentage of Th2 2D2 cells in the lung could in turn correlate with decreased disease severity when compared to OVA-induced AAI.

C57BL/6 mouse strain was used in a different AAI study which involved an adoptive transfer of 2×10^6 cells Th2 polarised antigen-reactive CD4⁺ cells obtained by isolating CD4⁺ cells from the spleens of OVA immunized mice (Hogan et al., 1998). The antigen in that study was given via the intranasal route for 8 days, every second day. That model was not characterised in detail though and was only done in IL-5-deficient mice without comparison to WT mice. The two main readouts from that study included BAL eosinophilia which was more severe compared to Th2 2D2 model used here and a detectable AHR. Previous work within the laboratory has used the same Th2 polarisation and AAI induction protocols and the same strain of mouse as in this project but a different antigen, OVA, and OVA₃₂₃₋₃₃₉-reactive CD4⁺ (OT-II) cells to drive the disease (Mackenzie, 2011). Comparing the two models, the lung pathology was strikingly more severe in Th2 OT-II-driven AAI with the eosinophil

contribution in BAL reaching ~80-90% compared to ~30% observed in the Th2 2D2-driven model. Moreover, the percentage and total number of Th2 OT-II cells in the lung upon OVA challenge were ~10-fold higher compared to the percentage and number of Th2 2D2 cells upon rMOG challenge. Interestingly, the frequency and total numbers of Th2 OT-II and Th2 2D2 cells in medLN were similar. This supports the hypothesis that Th2 polarised 2D2 cells may have a disadvantage in their ability to traffic into the lung or they are prone to death once they get into the tissue albeit in the presence of their cognate antigen. This could be potentially related to the fact that MOG is an autoantigen and host self-regulatory mechanisms could limit the pathogenicity of 2D2 cells at least in the context of Th2 type of inflammation.

In conclusion, the experiments described in this chapter demonstrated that Th2 polarised 2D2 CD4⁺ cells are capable of inducing moderate AAI upon airway challenge with rMOG which is characterized by lung eosinophilia, inflammatory infiltration of the lung and goblet cell hyperplasia. Increased production of Th2-associated cytokines and expression of GATA-3 by Th2 2D2 cells isolated from the antigen-challenged lung provide further evidence of Th2-mediated inflammatory response and a possible readout of how pathogenic Th2 2D2 cell function/phenotype can be modified by regulatory mechanisms.

3.3.3. A role for Foxp3⁺ cells in the control of lung inflammation in AAI

The importance of Foxp3⁺ cells in regulating lung inflammation in AAI was discussed in section 1.8.2.3. Multiple studies have demonstrated protective effects of CD4⁺ CD25⁺ cell transfer (Kearley et al., 2005, Leech et al., 2007) and increased disease severity upon CD25⁺ cell depletion prior to antigen challenge (Lewkowich et al., 2005, Leech et al., 2007). However, only a limited number of studies have investigated how endogenous Foxp3⁺ cell populations change during AAI and none of the studies assessed this in an adoptive transfer model of AAI. Leech et al. showed that during acute self-resolving AAI induced by HDM-derived Der p1 the percentages of Foxp3⁺ cells increase within the lung and medLN upon airway challenge (Leech et al., 2007). This increase was detectable four days after the last

antigen challenge and corresponded with the peak of disease. This elevated Foxp3⁺ cell frequency then started to decrease and returned to a steady-state level once the inflammation was fully resolved. This observation strongly suggested a role for Foxp3⁺ cells in limiting the pathology in that model which was confirmed by depletion studies (Leech et al., 2007). Here, kinetics study of rMOG-induced AAI revealed that the peak of lung inflammation occurred two days after the last antigen challenge and this corresponded with the highest frequency and absolute numbers of Foxp3⁺ cells in the lung, and with the highest numbers of Foxp3⁺ cells within medLN. The percentage of Foxp3⁺ cells decreased just two days after the peak and returned to a steady-state level between days 11 and 16 which also corresponded with disease recovery. This similarity in the kinetics of Foxp3⁺ cell changes during lung inflammation seen in both Der p1- and rMOG-induced models of AAI highlights how quickly Foxp3⁺ cells are able to respond to Th2-mediated inflammation and how effectively they can control it by transient changes in their proportion. Whether the function of Foxp3⁺ cells is affected, is as yet undetermined.

The increase in the total numbers of Foxp3⁺ in the lung tissue following allergen exposure has been also demonstrated in OVA-mediated AAI as well (Lu et al., 2011). However, it was not shown how it correlates with disease severity. Cell proliferation measured by BrdU incorporation performed in that study indicated that the majority of lung Foxp3⁺ cells actively proliferated *in situ*. Interestingly, the expansion of Treg within the inflamed CNS during MOG-induced EAE was also shown to be associated with rapid proliferation of Treg within the CNS rather than *de novo* induction of Foxp3⁺ cells (Korn et al., 2007, O'Connor et al., 2007). Whether elevated levels of Foxp3⁺ cells observed in the Th2 2D2-driven AAI model could be a result of rapidly proliferating nTreg or rather nTreg migration from the periphery or *de novo* induction of iTreg needs to be determined. Moreover, as the antigen-reactivity of Foxp3⁺ cells in AAI has never been investigated and it was reported that in EAE the CNS-derived Treg are enriched for myelin-reactive Treg (Korn et al., 2007, O'Connor et al., 2007), it would be valuable to know whether in the Th2 2D2-driven model of AAI there is an enrichment for antigen-reactive Foxp3⁺ cells as well.

The importance of host Foxp3⁺ cells in an adoptive Th2 cell transfer model has not been addressed before. Having established that Foxp3⁺ cell population increases in size following rMOG challenge in AAI and that this correlates with disease severity, it was decided to investigate the effects of depleting these cells. Most studies have previously used α -CD25 antibody to deplete regulatory CD4⁺ CD25⁺ cells (Lewkowich et al., 2005, Leech et al., 2007). However, as this model involved transfer of highly activated CD25⁺ Th2 2D2 cells, this method of depletion was not possible. Hence, Foxp3.LuciDTR-4 mice were used (Suffner et al., 2010). Foxp3⁺ cells were depleted using DTx prior to and during rMOG-induced AAI as it has been reported that their numbers do readily recover in the absence of DTx (Suffner et al., 2010). Ablation of Foxp3⁺ cells was not found to significantly change total BAL counts or eosinophil percentage, however it significantly increased lung infiltration scores leaving goblet cell hyperplasia unaffected. Moreover, it resulted in elevated percentages of CD4⁺ cells within the lung and slightly higher percentages of Th2 2D2 cells within the lung and medLN. These results partially disagree with previously published observations from a sensitisation model of HDM-mediated AAI in which an increase in total BAL cells and eosinophilia but no effect on lung infiltration were reported upon Foxp3⁺ cell depletion (Leech et al., 2007). This however, could be due to the use of a CD25 depleting antibody in that study, a different model of AAI and the antigen used. Interestingly, in line with results from the Th2 2D2-driven AAI model, selective Foxp3⁺ cell depletion using DTx during the sensitization phase was demonstrated to enhance lung infiltration but did not affect goblet cell hyperplasia in OVA-induced AAI model (Baru et al., 2010). The elevated production of Th2-associated IL-13 and GM-CSF by Th2 2D2 cells in the lung upon Foxp3⁺ cell depletion which was observed in rMOG-induced AAI was also reported in other models (Leech et al., 2007, Lewkowich et al., 2005, Baru et al., 2010).

An interesting observation made here following Foxp3⁺ cell depletion was the increased frequency of Foxp3⁺ cells within the transferred Th2 2D2 cell population. On the day of transfer, Foxp3⁺ cells constitute less than 1% of total Th2 2D2 cells. This percentage was found to increase to ~18% in the lung and to ~16% in the

medLN upon DTx treatment and rMOG challenge. However, it was only found to be significantly different in the medLN because of large variability within the groups. It is not known whether this increase is associated with expansion of the small percentage of Foxp3⁺ cells transferred within Th2 2D2 cell population or an active Foxp3 induction in Th2 2D2 cells. It has been reported previously that in Foxp3-depleted mice there is a homeostatic proliferation of the remaining Foxp3⁺ cells to ensure rapid recovery of Treg compartment (Suffner et al., 2010). Hence, an increased frequency of Foxp3⁺ cells within Th2 2D2 cell population upon DTx treatment could provide an additional compensatory mechanism for the depleted host Foxp3⁺ cells.

3.4. Concluding remarks

- Sensitization and subsequent airway challenge with rMOG does not induce AAI in WT or MOG-deficient mice.
- Adoptive transfer of Th2 polarised 2D2 CD4⁺ induces moderate lung inflammation upon rMOG challenge, characteristic of Th2-mediated AAI.
- Lung inflammation in rMOG-induced AAI is associated with increased frequency of 2D2 Th2 cells within the lung and medLN.
- The peak of lung inflammation in rMOG-induced AAI correlates with increased frequency and total numbers of Foxp3⁺ cells within the lung.
- Depletion of host Foxp3⁺ cell prior to and during AAI induction results in increased disease severity.

This rMOG-induced model of AAI provides a critical tool for testing the importance of Treg phenotype versus antigen-responsiveness in their ability to suppress T cell responses in different inflammatory conditions. The phenotype of Foxp3⁺ cells in different inflammatory contexts both *in vitro* and *in vivo* is discussed in Chapter 4, whereas therapeutic activity of Treg is the focus of Chapter 5.

4 Treg polarisation *in vivo* and *in vitro*

4.1. Introduction

Different T helper lineages are characterized by the expression of distinct transcription factors and chemokine receptors such as expression of T-bet and CXCR3 in Th1 cells or expression of GATA-3 and CCR4 in Th2 cells. Although Foxp3⁺ Treg have been shown to differentially express a variety of tissue-specific markers and homing molecules (Siewert et al., 2007, Kohm et al., 2002), it is unclear whether they can 'specialize' like effector CD4⁺ T cells (Teff) do in particular cytokine environments to maximise their suppressive potential towards differentiated Teff.

Evidence for Treg 'specialization' comes from the studies on the murine models of infection. Foxp3⁺ CD4⁺ cells have been shown to upregulate expression of T-bet during chronic infection with *Mycobacterium tuberculosis* (Koch et al., 2009). In that study T-bet was demonstrated to not only regulate homeostasis of Treg during Th1 responses *in vivo*, but also to induce CXCR3 expression in Treg which was important for their migration towards the site of inflammation and was associated with more effective suppression of Teff both *in vitro* and *in vivo*. A different study has shown that Foxp3 regulates expression of Th2-associated transcription factor IRF-4 in Treg and its ablation leads to an autoimmune lymphoproliferative disorder characterized by uncontrollable Th2 responses which can be detected in mice as soon as they reach 3-4 weeks of age (Zheng et al., 2009). It has therefore been suggested that Treg may be capable of utilizing certain components of transcriptional machinery involved in promoting the differentiation and function of a particular Teff lineage to efficiently control the corresponding type of the immune response.

Whether Treg can acquire different phenotypes during chronic inflammation observed in differentially mediated autoimmune and allergic diseases and how this correlates with their suppressive potential is currently unknown. In this project, based on evidence from the infection and homeostasis studies, it was hypothesized that

Treg derived from the CNS in Th1/Th17-mediated EAE and from the lung in Th2-mediated AAI will differ in their expression of Th-associated transcription factors and chemokine receptors and this will influence their ability to inhibit different types of Teff responses. It is important to note that both of these models represent self-resolving and relatively short inflammation period lasting over days to 2 weeks rather than months. Hence, it would be interesting to determine whether relatively short inflammation time is sufficient for Treg specialization.

4.1.1. Aims

The main aims of the experiments described in this chapter were to:

- 1) Investigate Foxp3⁺ CD4⁺ cell phenotype in the inflamed tissue versus peripheral organs in Th1/Th17-mediated EAE and in Th2-mediated AAI.
- 2) Assess whether nTreg can be polarised *in vitro* and hence recapitulate *in vivo* observations, and establish what conditions are most optimal.
- 3) Characterize the phenotype of iTreg upon TGF- β -mediated induction and how this is affected by polarising conditions *in vitro*.

4.1.2. Approach

Foxp3-GFP mice were used throughout the experiments in this chapter unless otherwise stated as they enabled identification of Foxp3⁺ cells without the need for the intracellular Foxp3 staining. Differences in the Foxp3⁺ cell phenotype in differentially mediated inflammatory diseases *in vivo* were first investigated. EAE was used as a model of Th1/Th17-mediated autoimmune inflammation and rMOG-induced AAI (described in Chapter 3) was used as a model of Th2-mediated allergic inflammation. Foxp3⁺ Treg were characterized in terms of expression of Th-associated transcription factors, chemokine receptors and activation markers and the phenotype of tissue-derived and peripheral Foxp3⁺ CD4⁺ cells was compared in both diseases.

To establish whether *in vivo* observations could be recapitulated *in vitro*, changes in nTreg phenotype were assessed upon their stimulation under Th1 or Th2 polarising conditions. As the numbers of nTreg available for polarisation were limited and significantly diminished after *in vitro* culture, nTreg expansion with α -CD3/ α -CD28 T cell expander Dynabeads and high IL-2 concentration under polarising conditions was utilized. In addition to nTreg, iTreg which provide an easy means of generating robust numbers of cells capable of effectively suppressing pathogenic T cell responses were also characterized. The phenotype of iTreg was assessed upon TGF- β -driven Foxp3 induction *in vitro*, and following their generation and re-stimulation under polarising conditions. The stability of Foxp3 expression in Treg manipulated *in vitro* was also addressed.

4.2. Results

4.2.1. Phenotypic analysis of Foxp3⁺ cells *in vivo*

4.2.1.1. Upregulated expression of T-bet and CXCR3 in Foxp3⁺ cells from inflamed CNS in EAE

To investigate the phenotype of Treg in a Th1/Th17-mediated inflammation, EAE was induced by immunization of Foxp3-GFP mice with MOG₃₅₋₅₅ in CFA (Figure 4.1A). On day 14 to 17 post-immunization as the animals were recovering the CNS, inguinal lymph nodes (ingLN) draining the site of immunization and spleen were harvested for the flow cytometric analysis (Figure 4.1B). Spleen of naïve mice was used as a control. The percentage of Foxp3⁺ CD4⁺ cells was increased to ~20-30% in the CNS compared to ~15% in ingLN and ~10% in the spleen (Figure 4.1C). The increase in the percentage of Foxp3⁺ CD4⁺ cells during the EAE recovery phase has been previously demonstrated and was shown to be driven by the CNS inflammation itself and to be critical for the disease resolution (McGeachy et al., 2005, O'Connor et al., 2007). The phenotype of Foxp3⁺ CD4⁺ cells isolated from the CNS was compared to that of peripheral Treg isolated from the EAE spleen and the naïve spleen. The expression of T-bet was found to be specifically upregulated in Treg derived from the inflamed CNS, whereas its expression in the EAE spleen and the control spleen was absent (Figure 4.1D). It is important to note that expression of T-

Tbet in Treg within the CNS was lower compared to its expression in effector CD4⁺ T cells from the same site (data not shown). Low GATA-3 expression was detectable Foxp3⁺ CD4⁺ cells isolated from the EAE CNS and spleen and the control spleen, and no difference in the expression levels was observed (Figure 4.1D).

The expression of the Th1-associated chemokine receptor CXCR3 was the highest on Treg isolated from the CNS (Figure 4.2A). CXCR3⁺ Treg comprised ~50-55% of total Treg compared to ~18-20% in the EAE and the naïve spleens. To assess cell activation status, expression levels of CD62L and CD44 were compared. All Treg isolated from the inflamed CNS were CD62L⁻ compared to ~10% and ~20% of CD62L⁺ Treg observed in the EAE and the naïve spleens, respectively (Figure 4.2B). The expression of CD44 was high on all Treg, however the highest levels were found within the CNS. Treg were also characterised in terms of CD73 expression which was shown to correlate with their suppressive potential (Kobie et al., 2006, Deaglio et al., 2007). CD73 was highly expressed on all Foxp3⁺ CD4⁺ cells, but its expression was the highest on Treg isolated from the inflamed CNS (Figure 4.2B).

It is important to note that a recently published study by the Vignali group has found that Foxp3-GFP mice used in this project have a perturbed Treg function in an inflammatory environment (Bettini et al., 2012). This is due to N-terminal GFP-fusion protein which is a hypomorph that leads to an altered Foxp3-driven epigenetic modification of genes in Treg from these mice compared to WT mice. It has been shown that this results in accelerated autoimmune diabetes (Bettini et al., 2012). Whether this has an effect on Treg phenotype and function in EAE is unknown and has to be determined.

These data demonstrate that Foxp3⁺ CD4⁺ cells derived from the inflamed CNS tissue have a distinct phenotype compared to the peripheral Treg and express higher levels of Th1-associated Tbet and CXCR3. Moreover, they are CD62L⁻ and CD44^{high} indicative of an activated phenotype and CD73^{high}. In order to see if this phenotype was associated specifically with a Th1/Th17-mediated response and not inflammation itself, the phenotype of Treg in Th2-mediated AAI was investigated.

4.2.1.2. Upregulated expression of GATA-3 and lack of T-bet in Foxp3⁺ cells from inflamed lung in AAI

As demonstrated in Chapter 3, an adoptive transfer of Th2 polarised 2D2 cells and airway challenge with rMOG induces Th2-mediated lung inflammation. This was shown to be associated with the increased frequency and total numbers of Foxp3⁺ CD4⁺ cells within the inflamed lung. The importance of Treg was demonstrated by the increased AAI severity when Foxp3⁺ cells were depleted. The AAI kinetics study indicated day 9 as the optimal time point to look at Foxp3⁺ cells. Following induction of AAI either in Foxp3-GFP or C57BL/6 mice, Foxp3⁺ CD4⁺ cells from the lung and the spleen were analysed by flow cytometry (Figure 4.3A, B). The spleen from PBS-challenged mice was used as a control.

No T-bet expression was found in the AAI lung, AAI spleen or the control spleen (Figure 4.3C). GATA-3 was expressed in Treg isolated from all sites. However, its expression was the highest on Foxp3⁺ cells derived from the inflamed lung (Figure 4.3C). It is important to note that increased GATA-3 expression in Foxp3⁺ cells from the lung was lower compared to GATA-3 expression in 2D2 Th2 cells isolated from the same site (data not shown). Although the MFI of CXCR3 expression of total Foxp3⁺ cells in the lung was increased when compared to the peripheral Foxp3⁺ CD4⁺ cells (data not shown), the percentage of CXCR3 highly positive Treg was ~10-12% and was similar to the frequency of CXCR3⁺ Treg in the AAI and the control spleens (Figure 4.4A). CD62L expression in the lung was negative in ~80% of Foxp3⁺ cells and was similar to Treg isolated from the AAI and the naïve spleens (Figure 4.4B). The expression of both CD44 and CD73 was high on all Treg but the highest expression levels were found on Treg derived from the AAI lung (Figure 4.4B).

Foxp3⁺ CD4⁺ cells isolated from the AAI lung were found to have a distinct phenotype from the peripheral Treg and from the CNS-derived Treg in EAE. They did not express T-bet and had upregulated expression of GATA-3. Unlike in EAE, there was no evidence of increased percentage of CXCR3⁺ Treg within the lung in AAI. In order to verify whether these results could be recapitulated *in vitro* and

establish what conditions were needed for this to occur, Treg stimulation and expansion under polarising conditions were next investigated.

4.2.2. Foxp3⁺ T cell manipulation *in vitro*

4.2.2.1. Lack of T-bet and low/intermediate levels of GATA-3 in Foxp3⁺ cells at a steady-state level

Ex vivo analysis of peripheral CD4⁺ T cells isolated from naïve Foxp3-GFP mice (Figure 4.5A) revealed that there is no T-bet expression in both Foxp3⁻ and Foxp3⁺ cells but low/intermediate levels of GATA-3 (Figure 4.5B). Moreover, GATA-3 expression was higher in Foxp3⁺ cells compared to Foxp3⁻ cells (Figure 4.5B).

4.2.2.2. nTreg upregulate T-bet and GATA-3 under Th1 and Th2 polarising conditions *in vitro*, respectively

Due to the low viability of sorted CD4⁺ Foxp3⁺ cells stimulated under polarising conditions (data not shown), it was decided to polarise whole splenocytes instead for the full phenotypic analysis. Moreover, a higher dose of 100U/ml IL-2 was used (compared to standard 10U/ml and 40U/ml IL-2 doses for Th1 and Th2 polarisations, respectively) as IL-2 was previously shown to be critical for the Treg survival (Sakaguchi et al., 2008). Cells isolated from the Foxp3-GFP mouse were stimulated with α -CD3 for 3 days under Th1 (IL-12, IL-18) or Th2 (IL-4, α -IL-12, α -IFN- γ) polarising conditions (Figure 4.6A). The percentage of Foxp3⁺ cells from a starting population of ~8-10% of total CD4⁺ cells decreased to 2-3% after 3-day culture reflecting rapid proliferation of Foxp3⁻ cells under polarising and stimulating conditions (data not shown). Foxp3⁻ cells were found to have an upregulated expression of T-bet when stimulated under Th1 polarising conditions, and upregulated expression of GATA-3 when stimulated under Th2 polarising conditions (Figure 4.6B&C). This is in agreement with previously well-established data on CD4⁺ T cell polarisation described in section 1.4. Foxp3⁺ cells were found to express higher levels of T-bet under Th1 conditions and higher levels of GATA-3 under Th2 conditions in a similar fashion to Foxp3⁻ cells (Figure 4.6B&C). Intermediate T-bet expression observed in Th2-polarised cells and GATA-3 expression in Th1-polarised

cells might be due to the high activation status of the cells. Overlays of transcription factor expression between Foxp3⁻ and Foxp3⁺ cells revealed that Foxp3⁻ cells expressed higher levels of T-bet and GATA-3 under Th1 and Th2 polarising conditions, respectively compared to Foxp3⁺ cells (Figure 4.6B).

It was concluded that IL-12 and IL-18 are sufficient to induce T-bet expression in nTreg, whereas IL-4 in the presence of blocking antibodies to IL-12 and IFN- γ is sufficient to induce GATA-3 expression. In addition to Th1 and Th2 conditions, Foxp3⁺ cell polarisation under Th17-skewing conditions was also investigated. However, due to the lack of use of exogenous IL-2 in the culture conditions (because of its ability to inhibit Th17 differentiation), Foxp3⁺ cells did not survive the 3-day stimulation (data not shown). It has to be noted that CD4⁺ Foxp3⁻-derived cytokines produced upon stimulation such as IFN- γ might be implicated in nTreg polarisation as the cell cultures were not sorted.

4.2.2.3. Expanding nTreg under polarising conditions

In addition to characterizing Foxp3⁺ cells stimulated under polarising conditions, it was investigated whether expansion of Treg under Th1 and Th2 conditions was possible, to obtain larger numbers of cells, not only for phenotypic but also functional analysis. Cells isolated from Foxp3-GFP mice were FACS sorted for CD4⁺ CD62L^{high} GFP⁺ cells and expanded *in vitro* with α -CD3/ α -CD28 T cell expander Dynabeads in the presence of high IL-2 concentration (1000U/ml) and/or Th1 or Th2 polarising conditions for 7 to 10 days (Figure 4.7A). The FACS sort was based on CD62L expression as it was shown previously that CD62L^{high} cells retain high Foxp3 expression during expansion, whereas CD62L^{low/-} Treg do not (Stephens 2009). Although the percentage of viable proliferating cells (judging from the light-scattering properties of the cells) was lower in nTreg expanded under Th1 or Th2 polarising conditions versus neutral conditions (Figure 4.7B), no significant differences were noted in Foxp3 expression or cell expansion (Figure 4.7C&D).

Phenotypic analysis revealed that Foxp3⁺ cells expanded under Th1 conditions upregulated expression of T-bet, whereas the cells expanded under either neutral or

Th2 conditions did not express T-bet (Figure 4.8A). GATA-3 expression was low on all expanded nTreg and was not promoted by Th2 polarising conditions (Figure 4.8A). CXCR3 was high on all cells. However, the highest expression was noted on nTreg expanded under Th1 conditions (Figure 4.8B). Such high CXCR3 expression observed in nTreg expanded under all conditions could be associated with high cell activation status and active cell proliferation.

4.2.2.4. TGF- β -induced Foxp3⁺ cells have a Th1-like phenotype

A different Treg subtype, TGF- β -induced Foxp3⁺ cells, can be easily generated *in vitro* in large numbers and have been demonstrated to effectively suppress pathogenic responses in various disease models (Chen et al., 2003, Zhang et al., 2010). Nevertheless, their phenotype upon induction and subsequent re-stimulation under different pro-inflammatory conditions has not been well characterized. It is unknown whether iTreg and nTreg can be ‘polarised’ in a similar fashion depending on the local cytokine environment but it has been established that they respond differently to pro-inflammatory cues in terms of the cytokines they produce (O'Connor et al., 2010) which may indicate differences in their phenotype.

To investigate the possible differences in iTreg phenotype, splenocytes from naïve Foxp3-GFP mice were FACS sorted for GFP⁺ CD4⁺ CD62L^{high} cells and stimulated for 5 days with α -CD3/ α -CD28 in the presence of IL-2 and TGF- β (Figure 4.9A). The induction efficiency of Foxp3 under these conditions varied between 85-95% of total cells depending on the experiment (Figure 4.9B). Following induction, iTreg were found to express high levels of T-bet which agrees with previously reported observations (O'Connor et al., 2010) and contrasts with very low T-bet expression by nTreg isolated from naïve mice (Figure 4.9C). GATA-3 was expressed at intermediate levels and was similar in iTreg and nTreg (Figure 4.9C). Approximately 55-60% of iTreg were found to be CXCR3⁺ compared to ~20% of CXCR3⁺ nTreg (Figure 4.10A). In terms of activation markers, both CD62L⁺ and CD62L⁺ cells were found within iTreg populations compared to the majority of nTreg being CD62L⁺ (Figure 4.10B). Both iTreg and nTreg expressed high levels of CD44, however as the cells were stained in separate experiments it is difficult to conclude whether

expression of CD44 was higher on iTreg as it appears to be. The expression of CD73 was high on both iTreg and nTreg (Figure 4.10B).

4.2.2.5. Induction of iTreg under polarising conditions

To investigate how the iTreg phenotype is affected by pro-inflammatory cytokines, iTreg were first induced in the presence of polarising conditions (Figure 4.11A). IL-12 and IL-2 on their own did not induce Foxp3 expression and the simultaneous addition of TGF- β was necessary for this to occur (Figure 4.11B&C). Addition of titrated concentrations of IL-12 was not found to affect Foxp3 induction nor T-bet expression by iTreg on day 5 of culture (Figure 4.11B&C). Experiments with simultaneous addition of IL-12 and IL-18 or IFN- γ gave similar results (data not shown). Thus T-bet expression is intrinsic to the iTreg program, but cannot be elevated by exogenous IL-12 during iTreg induction.

As IL-4 critical for Th2 differentiation was previously reported to inhibit expression of Foxp3 (Dardalhon 2008), it was decided to use titrated concentrations of IL-4 to establish the dose that would allow for effective iTreg induction. Addition of IL-4, α -IL-12 and α -IFN- γ without the presence of TGF- β did not induce Foxp3 expression and a dose dependent Foxp3 induction was observed with varying concentrations of IL-4 in the presence of TGF- β (Figure 4.12A&B). Expression of GATA-3 in iTreg was intermediate and remained unaffected by the presence of IL-4 in iTreg culture conditions (Figure 4.12A). Lower than usual GATA-3 expression in the control Th2 sample might be due to the inefficient induction of Th2 phenotype using sorted CD4⁺ cells without the presence of APC.

Investigation into individual effects of α -IL-12 and α -IFN- γ on IL-4-mediated inhibition of Foxp3 induction revealed that α -IFN- γ but not α -IL-12 opposed the inhibitory effect of IL-4 (Figure 4.13C). It has been shown that IFN- γ is produced in substantial amounts during iTreg induction (O'Connor et al., 2010) and hence blocking it may provide an advantage to the Foxp3 expression. Addition of α -IFN- γ without IL-4 did not promote Foxp3 induction and this could be due to already very efficient Foxp3 induction of ~95%.

4.2.2.6. Re-stimulation of iTreg under polarising conditions

iTreg have been demonstrated to effectively suppress pathogenic T cell responses *in vivo* when given prior to disease induction (Chen et al., 2003, Zhang et al., 2010). Little is known, however, about their ability to suppress on-going inflammation. Hence, it was decided to investigate the effects of pro-inflammatory cytokines on iTreg phenotype during their re-stimulation under polarising conditions *in vitro*. iTreg were induced in the presence of TGF- β and IL-2 for 5 days followed by FACS sorting for pure GFP⁺ cells and subsequent 3-day re-stimulation under different conditions (Figure 4.13A). Re-stimulation of iTreg in the presence of TGF- β +/- IL-2 resulted in ~90% of iTreg retaining their expression of Foxp3 compared to ~55% in the presence of IL-2 only (Figure 4.13B&C). When iTreg were re-stimulated in the presence of Th1 or Th2 polarising conditions they maintained ~10-15% of Foxp3 expression, however simultaneous addition of TGF- β effectively prevented Foxp3 loss (Figure 4.13B&C).

In terms of iTreg phenotype, re-stimulation under Th1 conditions without the presence of TGF- β diminished T-bet expression in Foxp3⁺ iTreg whereas under Th2 conditions abrogated it completely (Figure 4.14). The addition of TGF- β did not affect T-bet expression which was maintained in iTreg re-stimulated under both Th1 and Th2 conditions. GATA-3 expression was intermediate in iTreg re-stimulated under all conditions with TGF- β present and inhibited in its absence (Figure 4.14).

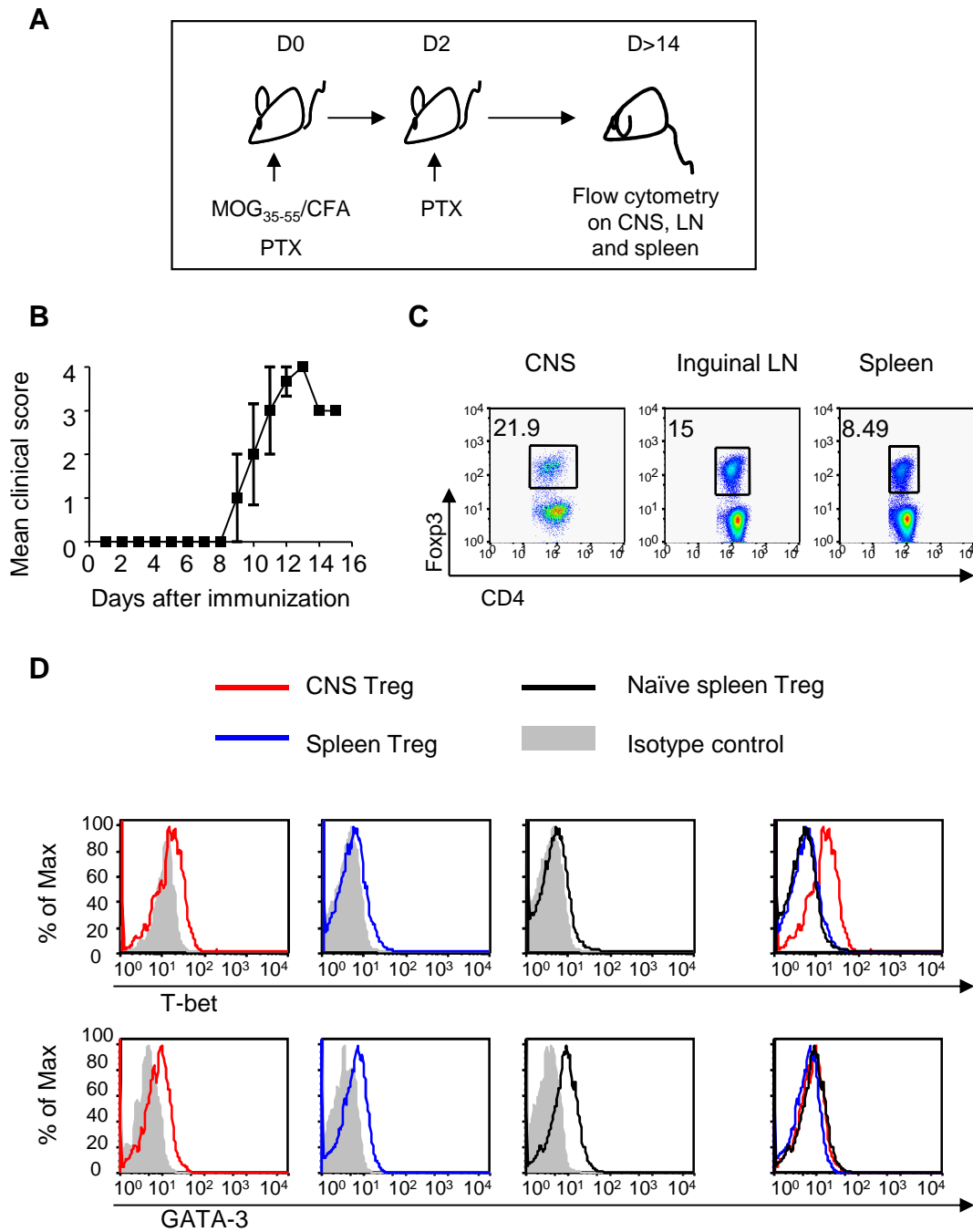


Figure 4.1 Treg derived from the inflamed CNS in EAE have an upregulated expression of T-bet.

EAE was induced in Foxp3-GFP mice by immunization with MOG₃₅₋₅₅ in CFA and two consecutive doses of PTX. Mice were harvested during recovery phase on days 14-17. (A) Induction protocol. (B) Mean clinical scores of EAE. (C) Representative percentages of Foxp3⁺ CD4⁺ cells in the CNS, inguinal lymph nodes and spleen of EAE mice. (D) Representative histograms showing expression of T-bet and GATA-3 in Treg isolated from the EAE CNS and spleen and from a naïve spleen as a control. Panel on the right shows overlays. Solid lines mark the staining and the shaded area is an isotype control. Data are representative of 3 experiments, $n \geq 4$

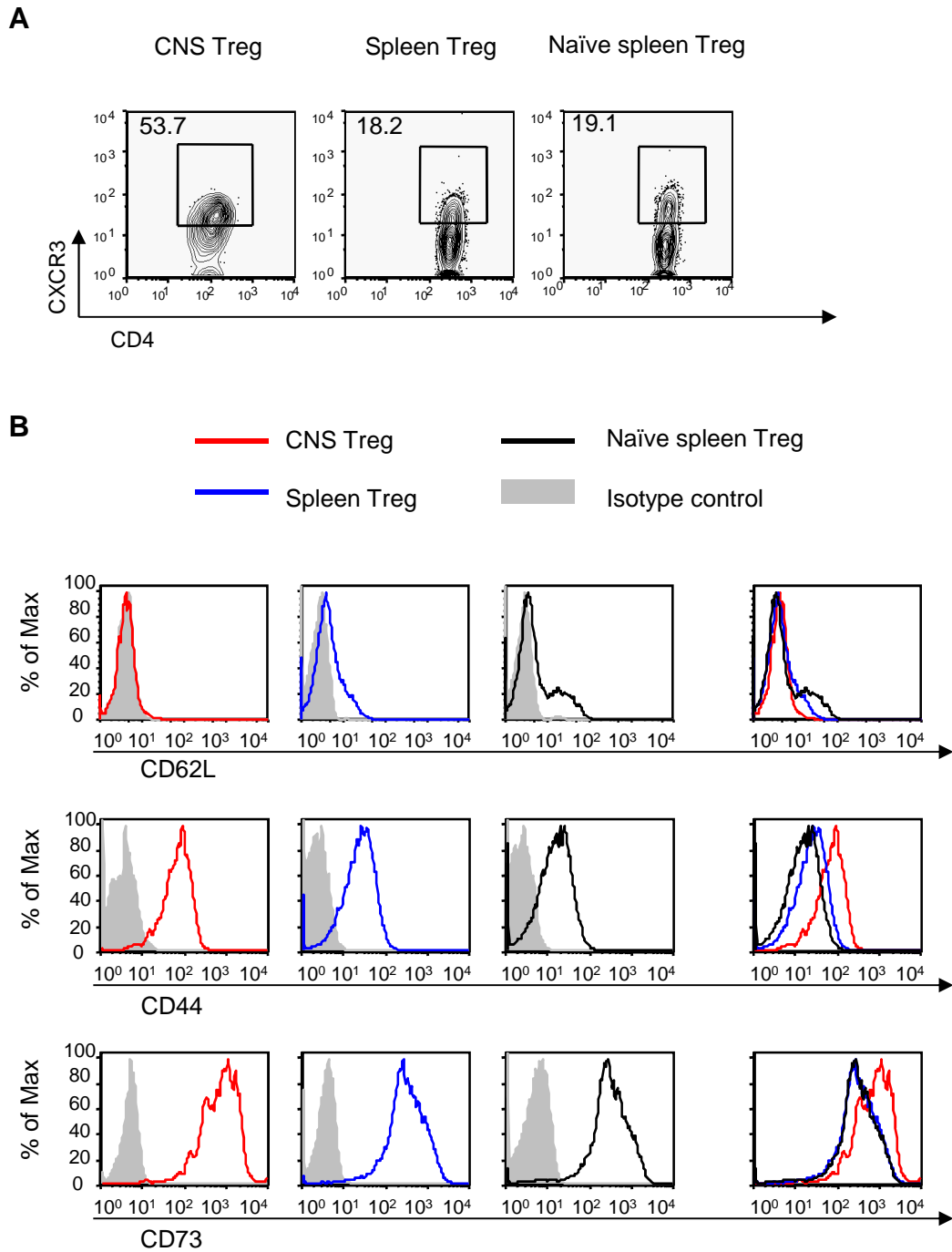


Figure 4.2 Treg derived from the inflamed CNS in EAE express high levels of CXCR3 and CD73 and have an activated phenotype.

Experimental scheme is shown in Figure 4.1. (A) Representative flow cytometry plots showing expression of CXCR3 and (B) representative histograms showing expression CD62L, CD44 and CD73 in Treg isolated from EAE CNS, spleen and a naïve spleen as a control. Solid lines mark the staining and the shaded area is an isotype control. Data are representative of 3 experiments, $n \geq 4$.

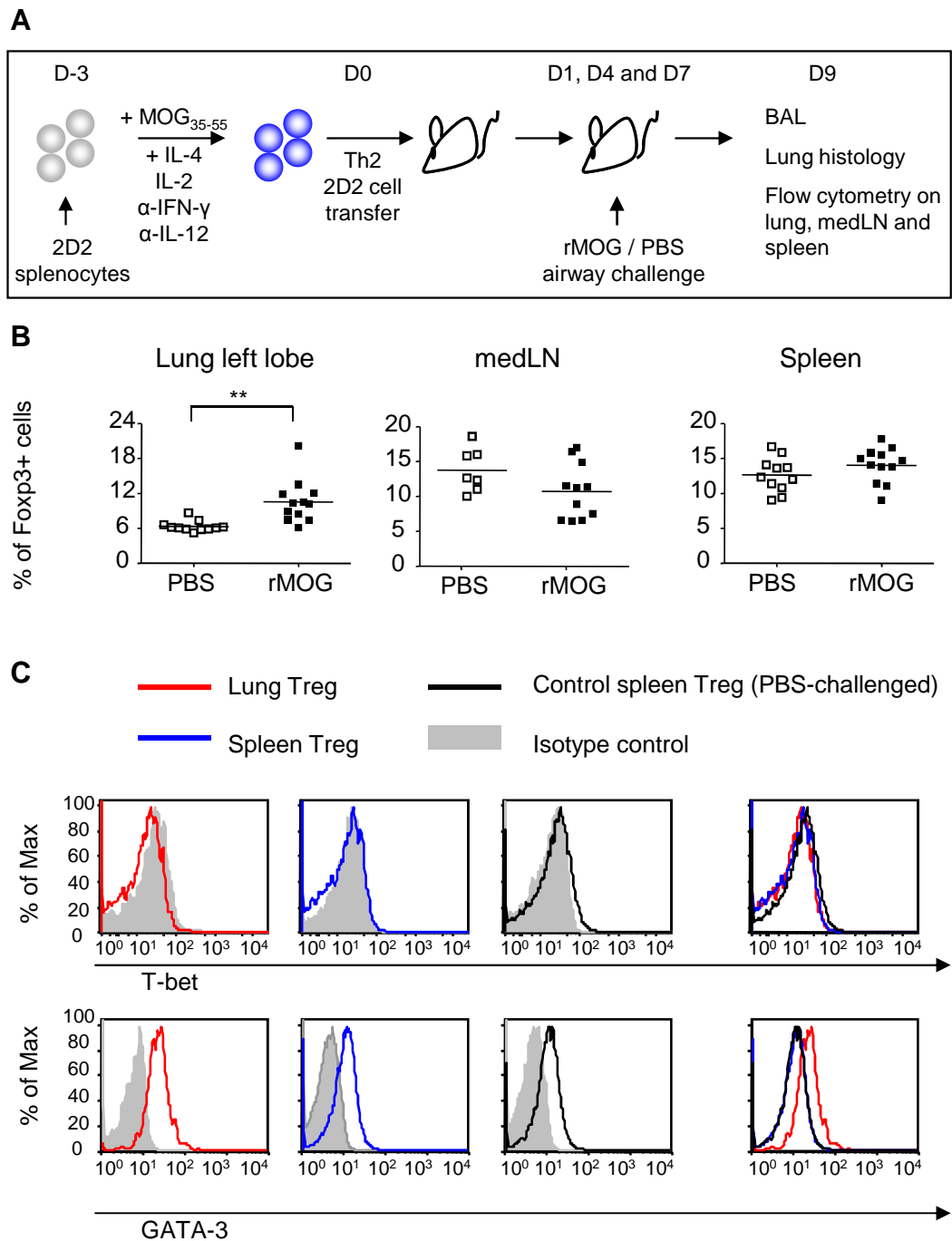


Figure 4.3 Treg derived from the inflamed lung in AAI do not express T-bet and have an upregulated expression of GATA-3.

AAI was induced in either Foxp3-GFP or C57BL/6 mice by an adoptive transfer of Th2 polarised 2D2 cells and airway challenges with rMOG. On day 9 the lung, medLN and spleen were harvested and analysed by flow cytometry. (A) Experimental scheme. (B) Representative histograms showing expression of T-bet and GATA-3 in Treg isolated from the AAI lung and spleen, and from the PBS-challenged spleen as a control. Panel on the right shows overlays. Solid lines mark the staining and the shaded area is an isotype control. Data are representative of 3 experiments, $n=4-5$. $**p<0.01$ as determined by an unpaired t-test.

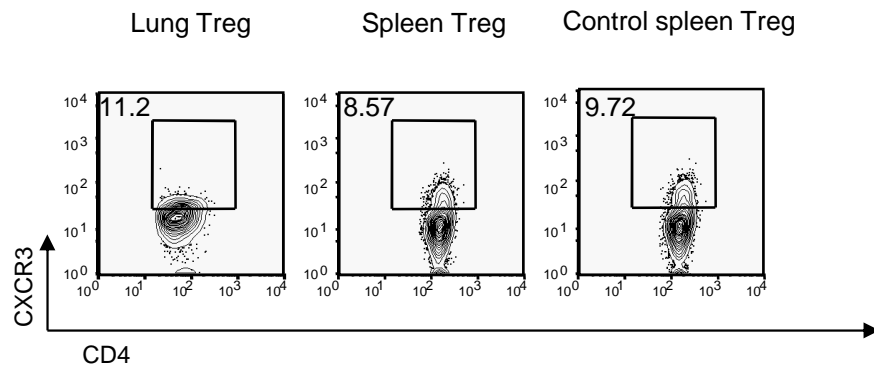
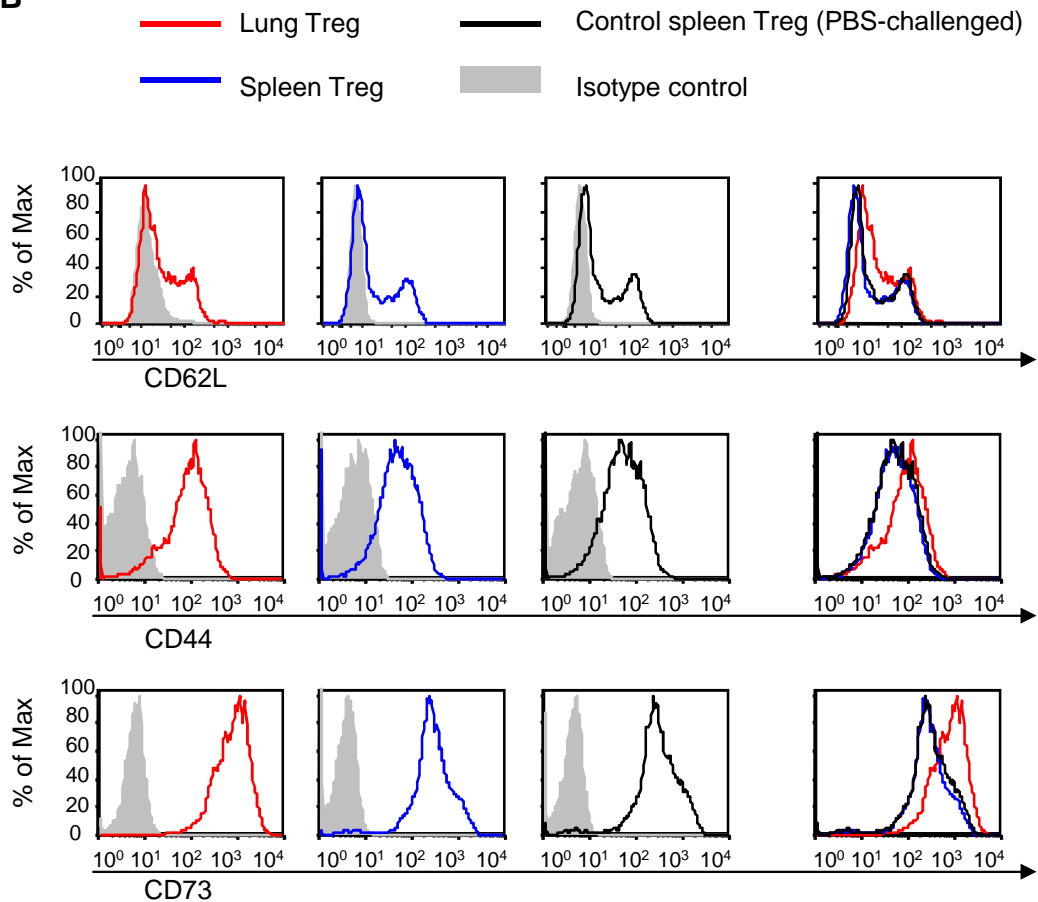
A**B**

Figure 4.4 Treg derived from the inflamed lung in AAI express steady-state levels of CXCR3, but are CD73^{high} and have an activated phenotype.

Experimental scheme is shown in Figure 4.3. (A) Representative flow cytometry plots showing expression of CXCR3 and (B) representative histograms showing expression CD62L, CD44 and CD73 in Treg isolated from the AAI lung, spleen and a PBS-challenged spleen as a control. Solid lines mark the staining and the shaded area is an isotype control. Data are (A) from one experiment, n=4 are (B) are representative of 3 experiments, n=4-5.

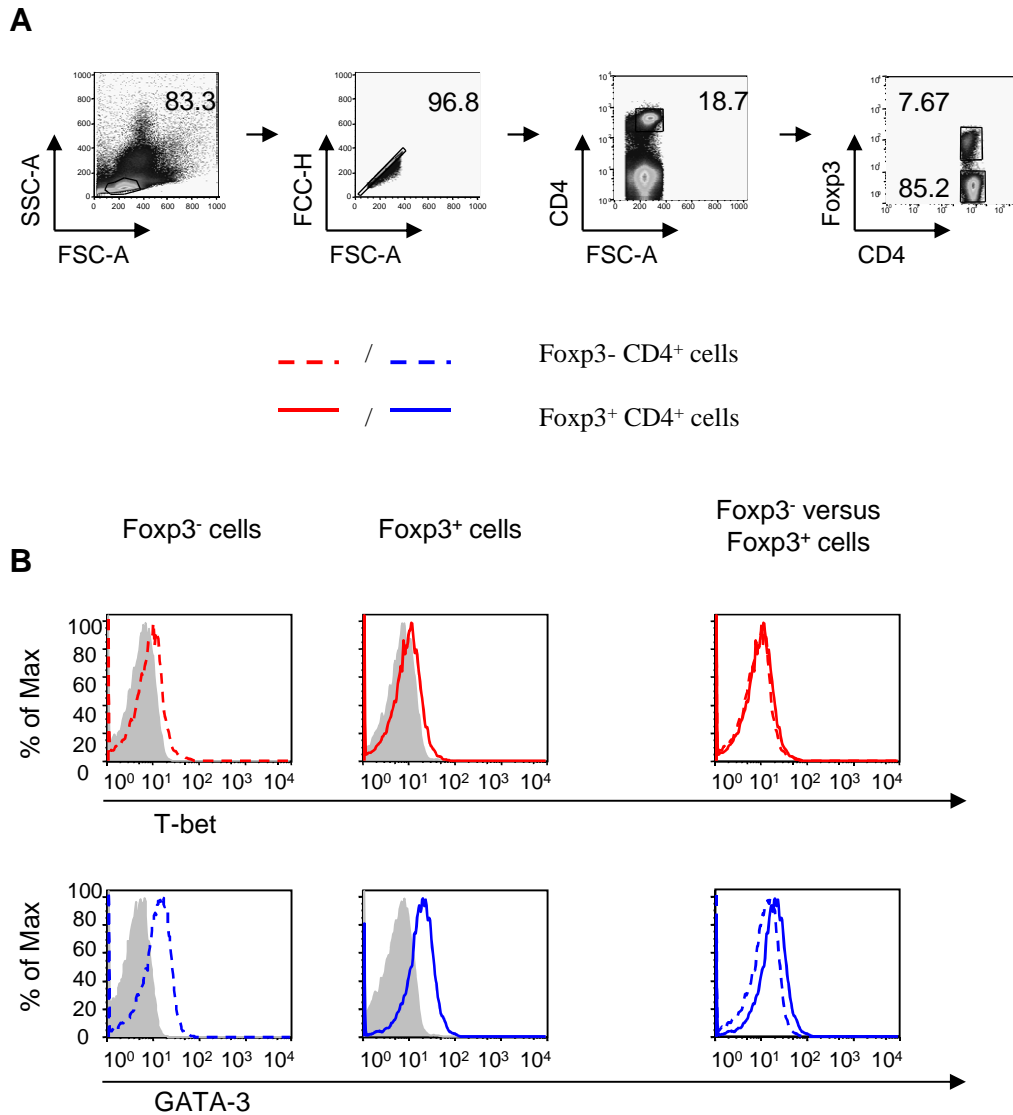


Figure 4.5 Foxp3⁺ CD4⁺ cells at a steady state are T-bet⁺ but express low/intermediate levels of GATA-3.

Whole splenocytes or FACS sorted GFP⁺ and GFP⁻ cells from Foxp3-GFP mouse were analysed by flow cytometry. (A) Representative flow cytometry plots showing the gating strategy for Foxp3⁺ and Foxp3⁻ CD4⁺ cell populations. (B) Representative histograms T-bet and GATA-3 expression including overlays between Foxp3⁻ and Foxp3⁺ cells. Red and blue solid (Foxp3⁺ cells) and dashed (Foxp3⁻ cells) lines mark the staining and a shaded area is an isotype control. Data are representative of 4 experiments, n=4-6 pooled.

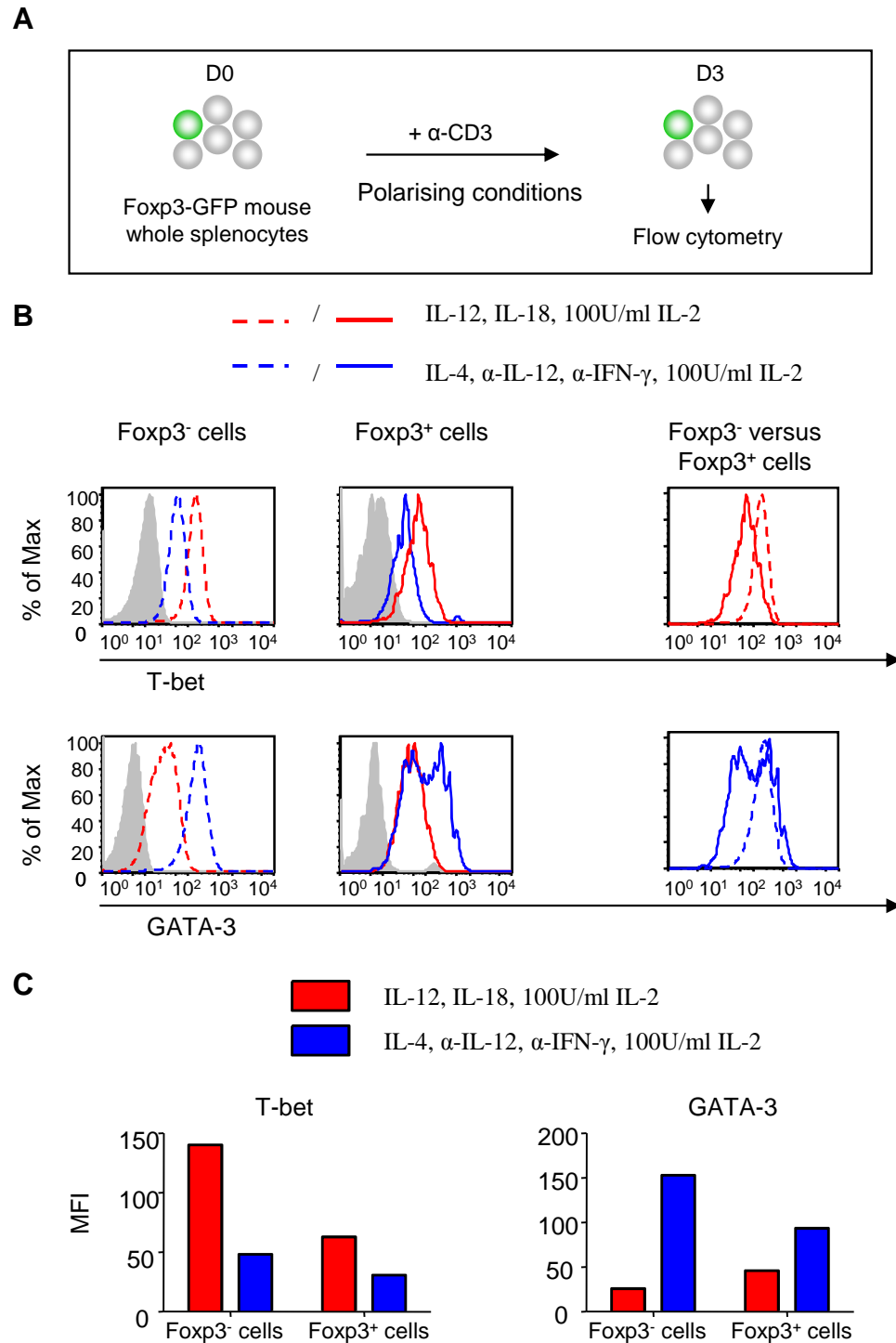


Figure 4.6 Foxp3⁺ CD4⁺ cells stimulated *in vitro* upregulate T-bet or GATA-3 expression under Th1 or Th2 polarising conditions, respectively.

Splenocytes from Foxp3-GFP mouse were stimulated with α -CD3 under Th1 or Th2 polarising conditions for 3 days followed by flow cytometry analysis. The gating strategy is shown in Figure 4.5. (A) Experimental scheme. (B) Representative histograms of T-bet and GATA-3 expression including overlays between Foxp3⁺ and Foxp3⁻ cells. (C) Mean fluorescence intensity (MFI) of T-bet and GATA-3 expression. Dashed lines represent Foxp3⁻ cells and solid lines Foxp3⁺ cells. Data are representative of 3 experiments.

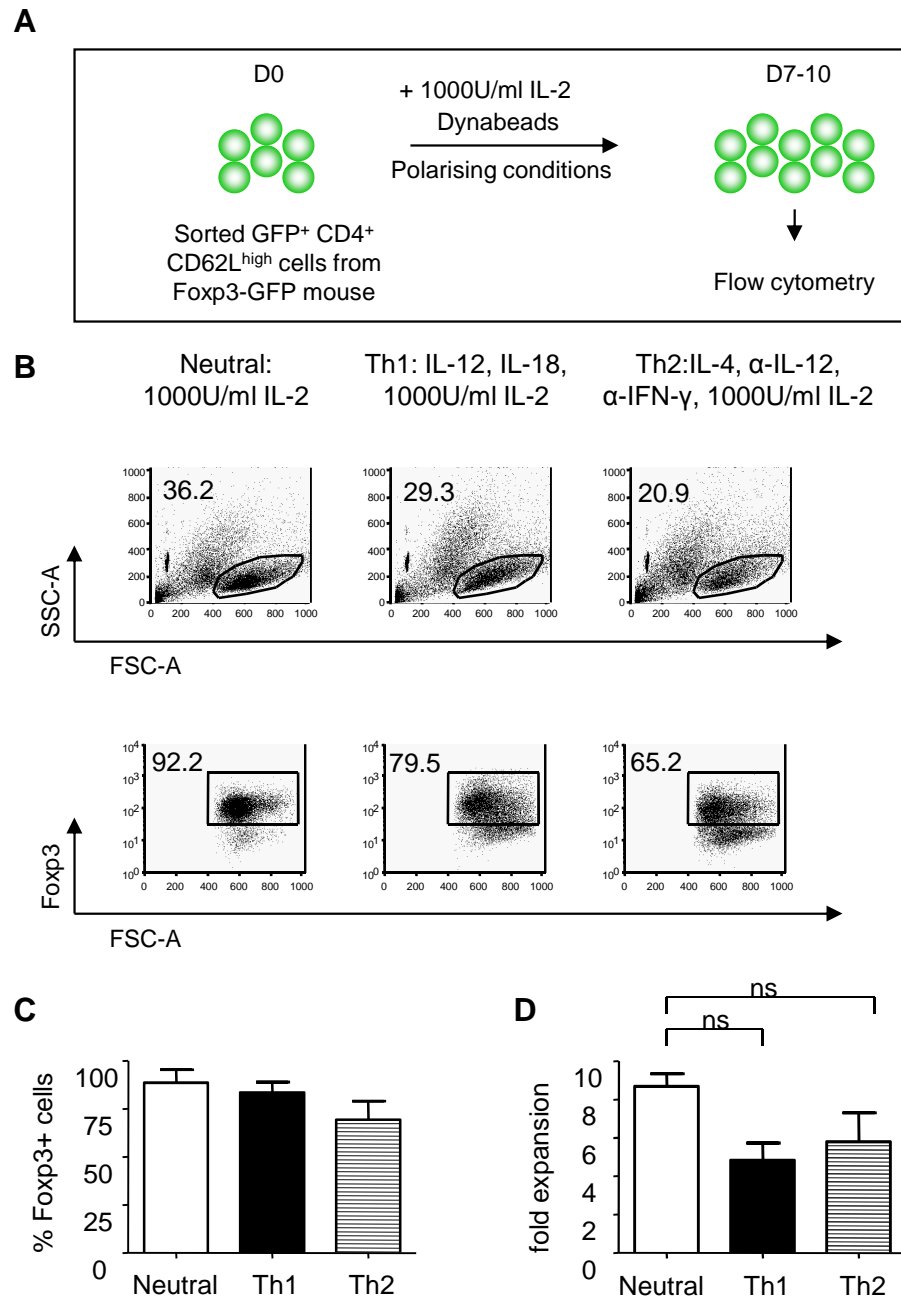


Figure 4.7 Expansion of nTreg under polarising conditions.

Cells from Foxp3-GFP mouse were FACS sorted for GFP⁺ CD4⁺ CD62L^{high} cells and expanded *in vitro* with Dynabeads in the presence of 1000U/ml IL-2 only (Neutral) or with the addition of Th1 or Th2 polarising conditions for 7 to 10 days. (A) Experimental scheme. (B) Representative flow cytometry plots showing forward (FSC) and side light-scattering properties (SSC) of cells and Fxp3 expression following expansion. (C) Percentage of Fxp3⁺ cells and (D) fold expansion of total cells. Data are representative of 3 experiments (B). Data are pooled from 3 experiments (C&D) Data are shown as mean \pm SEM. ns = not significant as determined by a one-way variance (ANOVA) using Tukey's multiple comparison test.

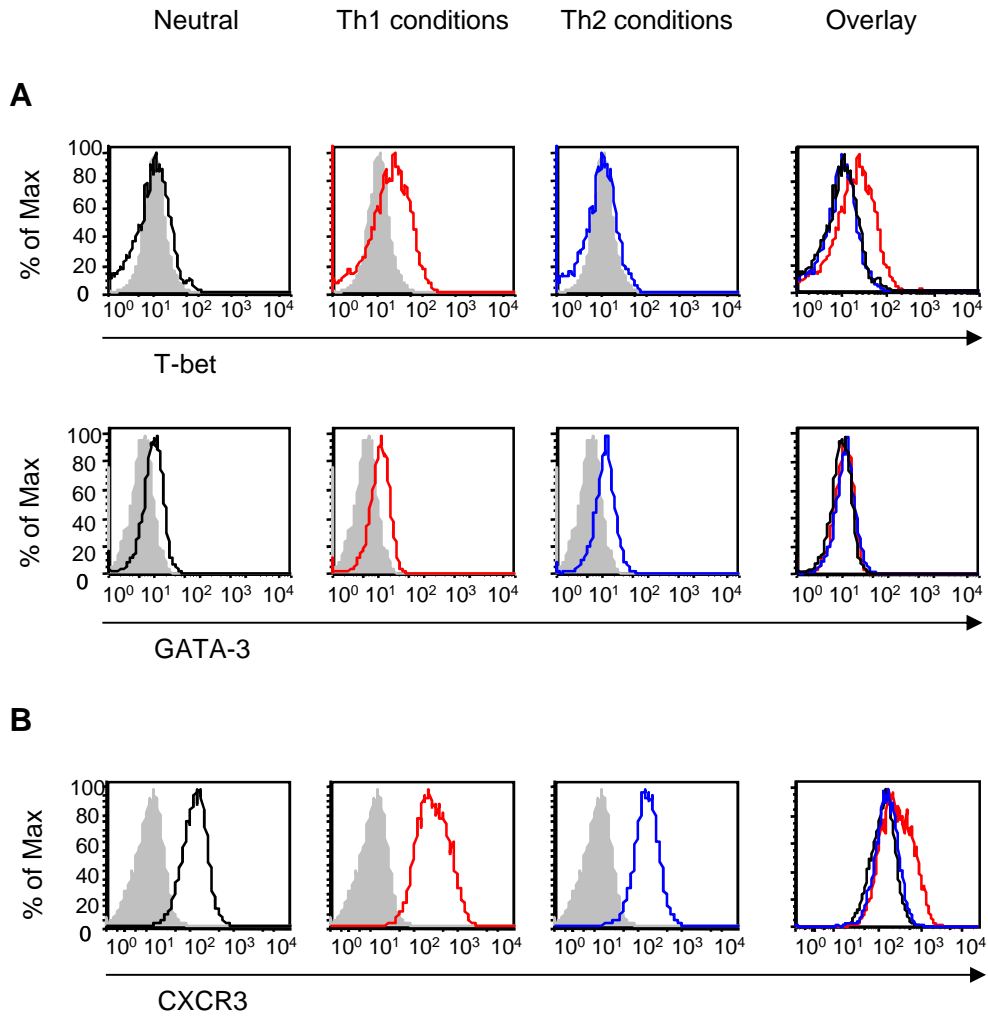


Figure 4.8 nTreg expanded under Th1 polarising conditions upregulate expression of T-bet and CXCR3 but maintain GATA-3.

Experimental scheme and gating strategy is shown in Figure 4.7. (A) Representative histograms of T-bet and GATA-3 expression and (B) CXCR3 expression in nTreg expanded under different polarising conditions. Data are representative of 2 experiments.

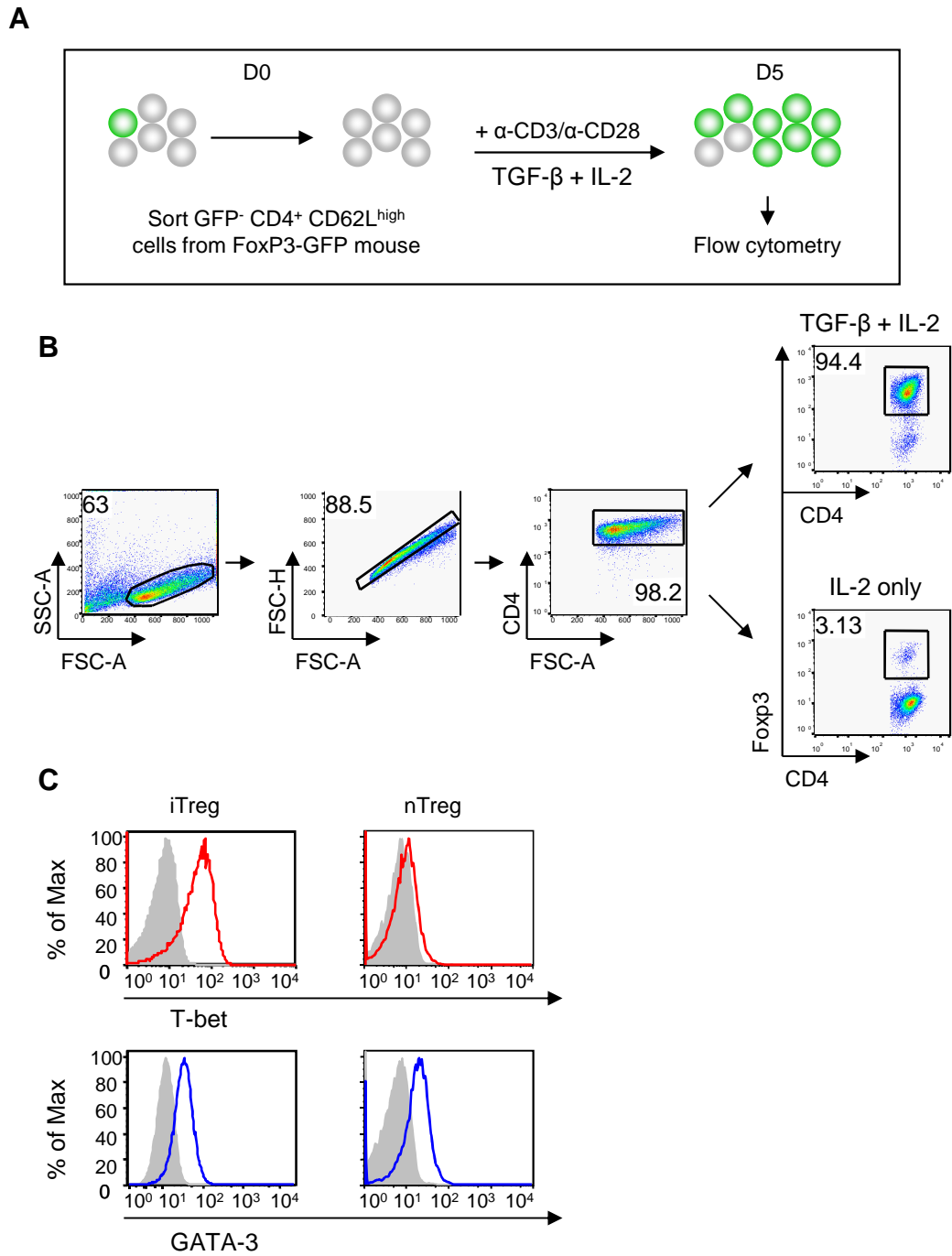


Figure 4.9 Generation and phenotype of iTreg.

Splenocytes isolated from Foxp3-GFP mouse were sorted for GFP⁺ CD4⁺ CD62L^{high} cells using FACS and stimulated in the presence or absence of TGF-β and IL-2 for 5 days. (A) Experimental scheme. (B) Representative flow cytometry plots showing the gating strategy for Foxp3⁺ CD4⁺ cells and the average Foxp3 induction efficiency. IL-2 only was used as a control. (C) Representative histograms showing the expression of T-bet and GATA-3 in iTreg versus naïve nTreg. nTreg staining was done in a different experiment. Red line marks expression of T-bet, blue line marks expression of GATA-3 and the shaded area is an isotype control. Data are representative of at least 3 experiments.

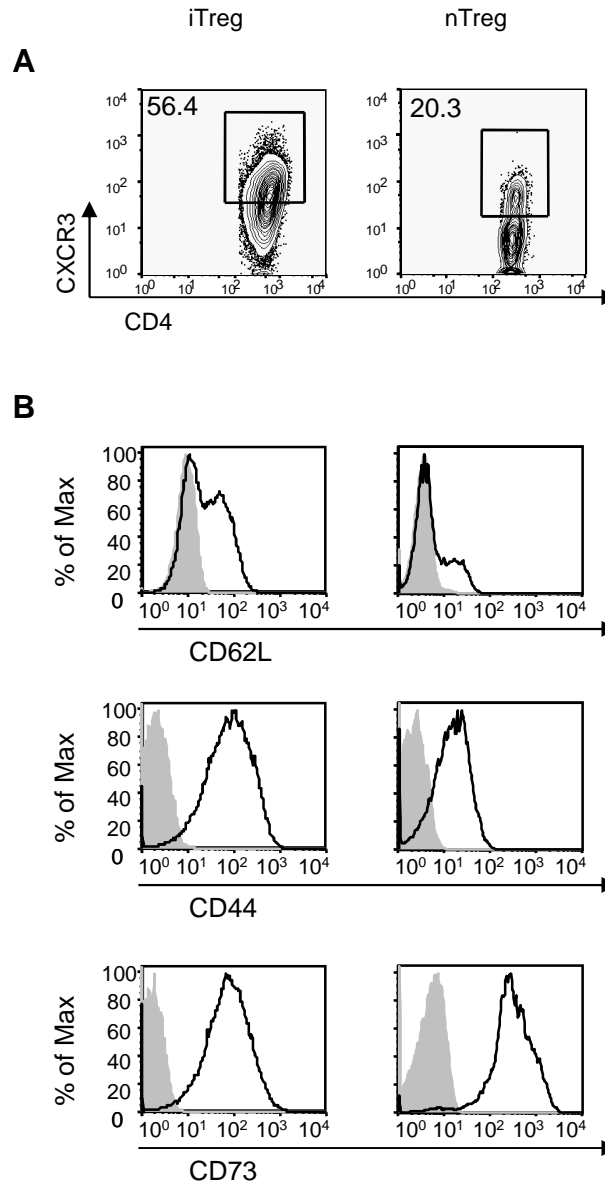


Figure 4.10 Phenotype of *in vitro* generated iTreg.

Experimental scheme and the gating strategy is shown in Figure 4.9. (A) Representative flow cytometry plots showing the expression of CXCR3 in iTreg versus naïve nTreg. (B) Representative histograms showing the expression of CD62L, CD44 and CD73 in iTreg versus naïve nTreg. nTreg staining was done in a different experiment. Black line marks the staining and the shaded area is an isotype control. Data are representative of at least 3 experiments.

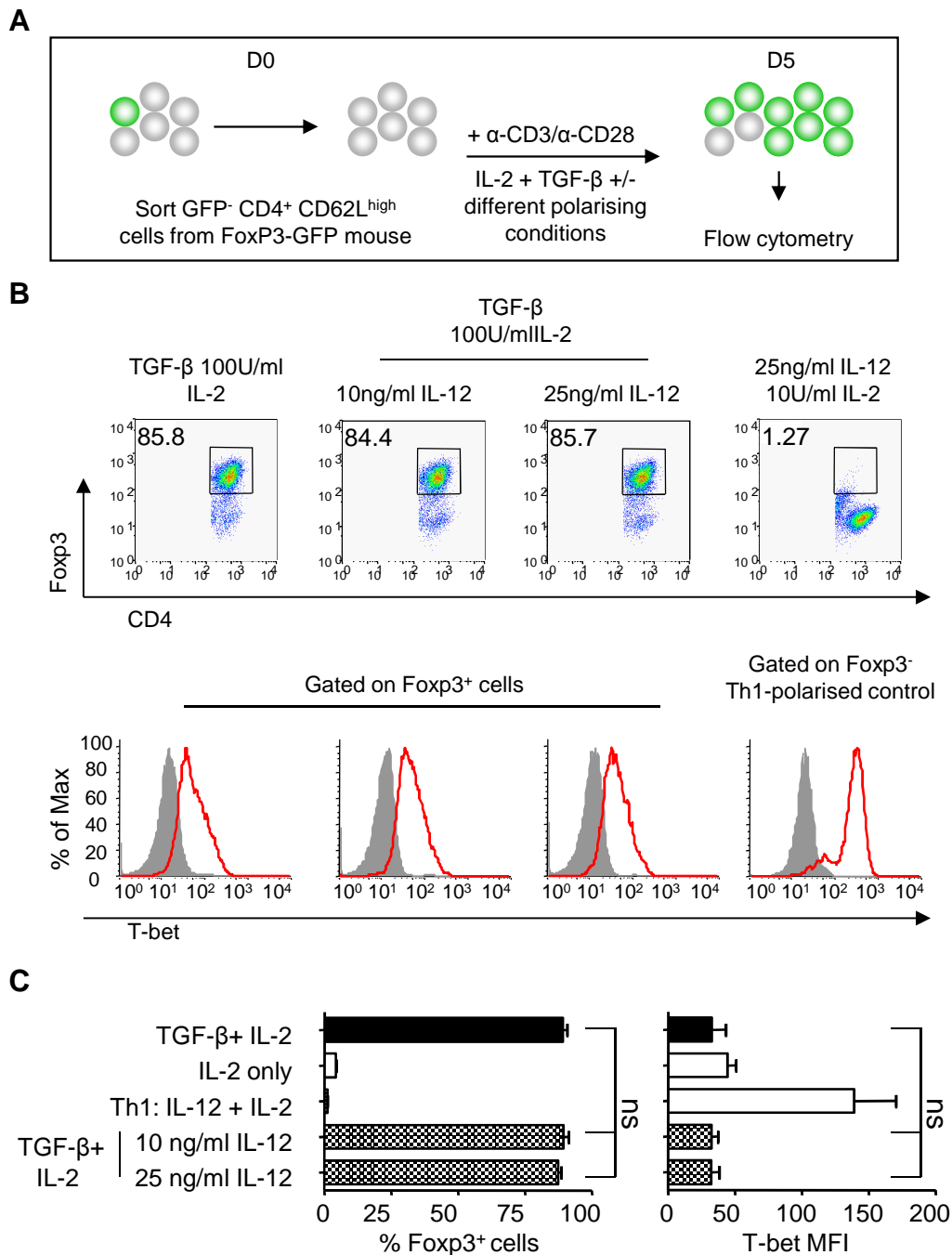


Figure 4.11 IL-12 does not affect Foxp3 induction and T-bet expression in differentiating iTreg.

Sorted GFP⁻ CD4⁺ CD62L^{high} cells were stimulated under different polarising conditions with or without TGF-β for 5 days followed by flow cytometric analysis. (A) Experimental scheme. (B) Representative flow cytometric plots showing Foxp3 expression and histograms of T-bet expression of cells cultured under different conditions. (C) Percentages of Foxp3⁺ cells (of total cells) and MFI of T-bet expression (gated on Foxp3⁺ cells or Foxp3⁻ cells for an IL-2 only and Th1-polarised control). Data are shown as mean ± SEM. Data are representative of 2 experiments. ns=not significant as determined by a one-way (ANOVA) analysis of variance between TGF-β + IL-2 +/- 10ng/ml and 25ng/ml IL-12.

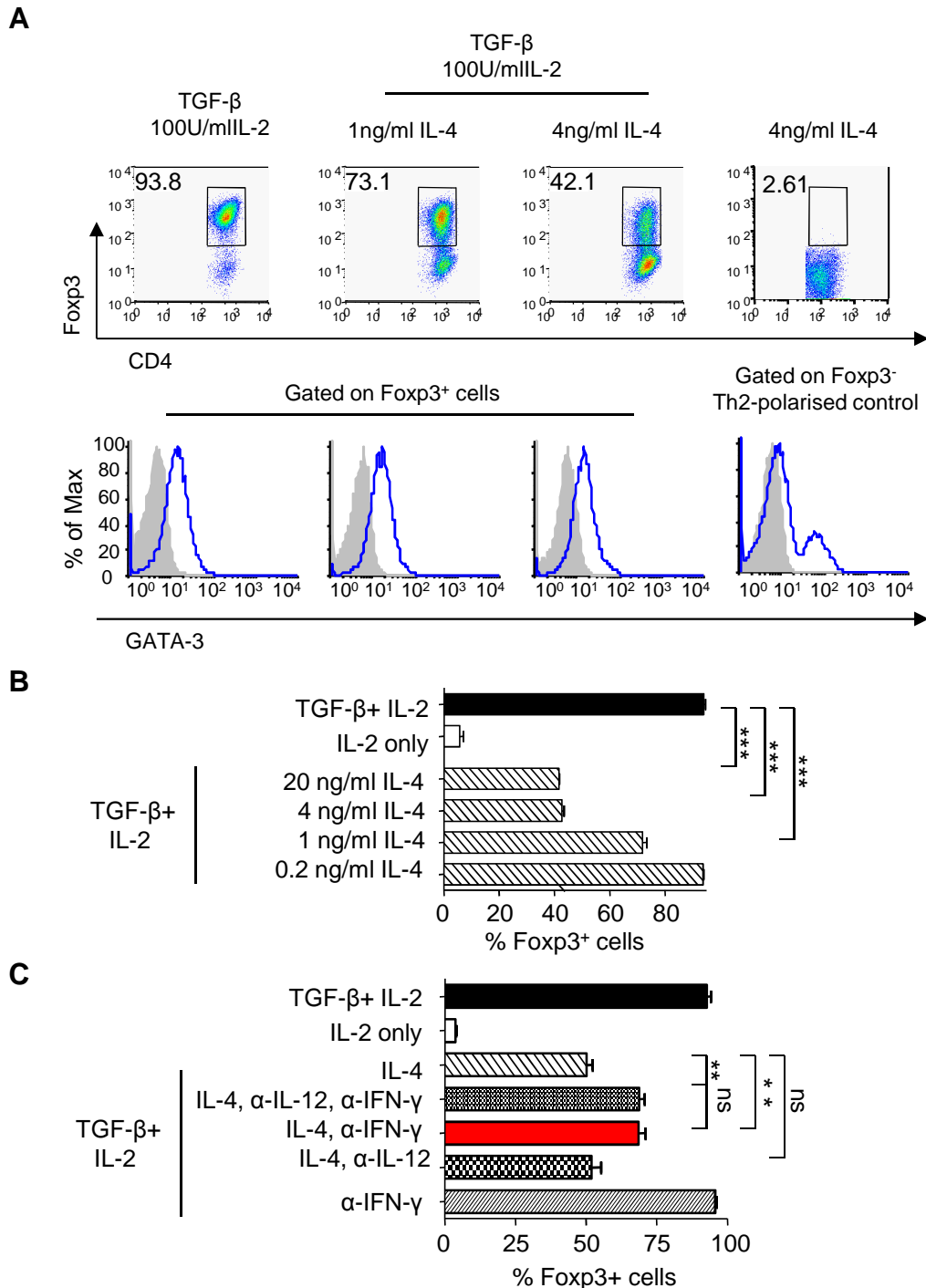


Figure 4.12 IL-4 inhibits Foxp3 induction in a dose-dependent manner and does not upregulate GATA-3 expression in iTreg at the time of induction.

Experimental approach is shown in Figure 4.11. (A) Representative flow cytometric plots showing Foxp3 expression and histograms of GATA-3 expression of cells cultured under different conditions. (B) Percentages of FoxP3 cells of all cells with varying concentrations of IL-4. (C) Percentages of Foxp3⁺ cells of all cells with α -IL-12 and α -IFN- γ added. Data are shown as mean \pm SEM. Data are representative of 2 experiments. ** p <0.1, *** p <0.01 as determined by a one-way (ANOVA) analysis of variance.

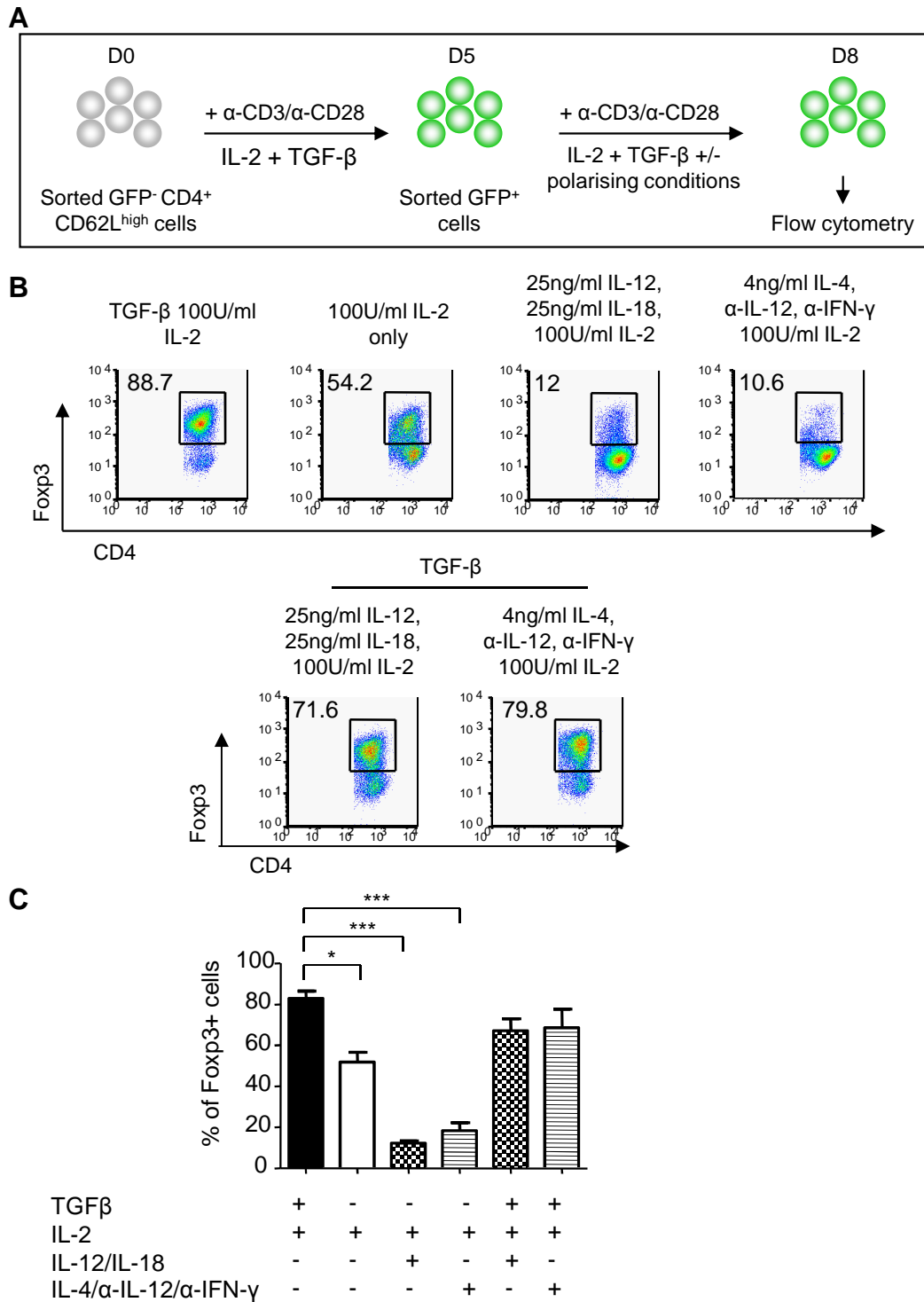


Figure 4.13 Re-stimulation of iTreg under polarising conditions leads to rapid loss of Foxp3 expression which is prevented by exogenous TGF-β.

iTreg were generated as described in Figure 4.9, FACS sorted for GFP⁺ cells and re-stimulated with α-CD3/α-CD28 for 3 days under different polarising conditions. (A) Experimental design. (B) Representative histograms of Foxp3 expression and (C) percentages of Foxp3⁺ iTreg re-stimulated under different conditions. Data are (B) representative of 3 experiments or (C) are pooled from 3 experiments. *p<0.5, ***p<0.01 as determined by a one-way (ANOVA) analysis of variance.

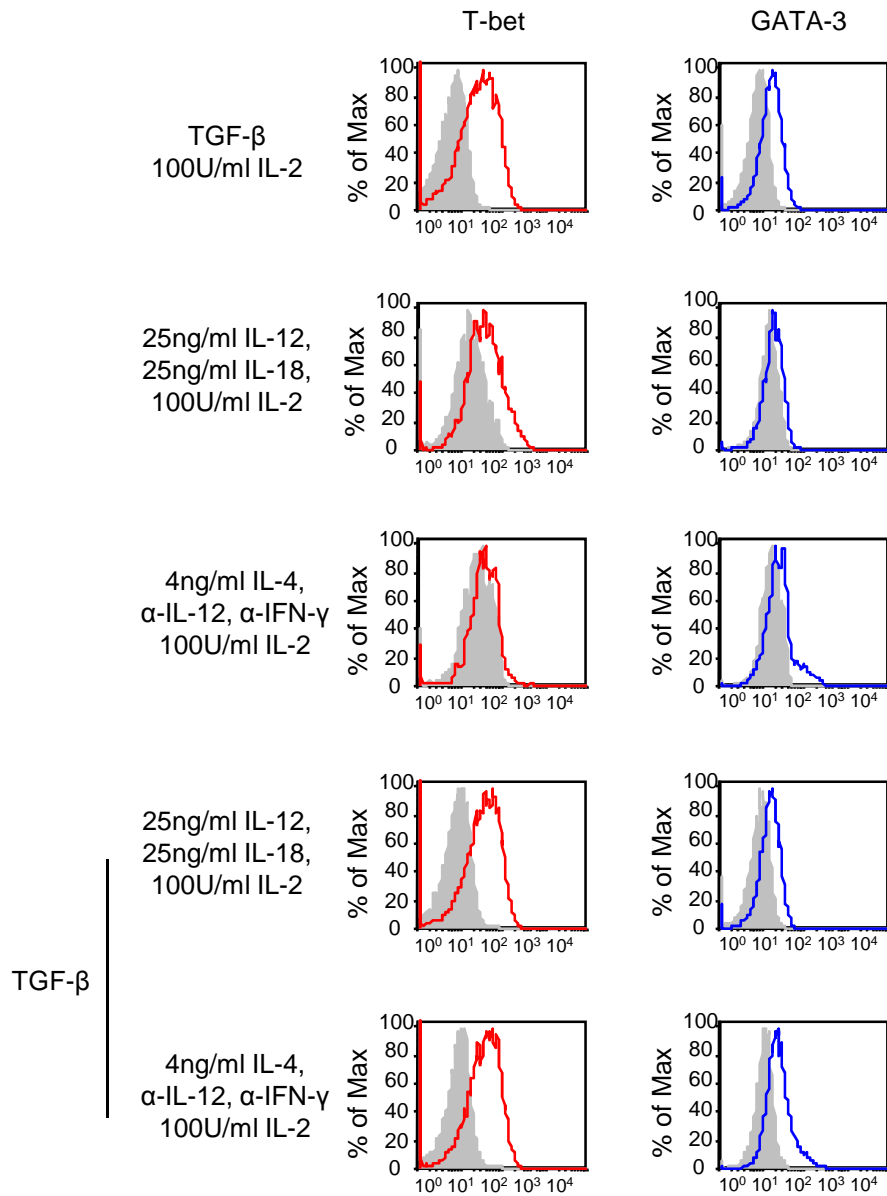


Figure 4.14 iTreg re-stimulated under Th1 or Th2 polarising conditions do not upregulate expression of T-bet or GATA-3, respectively.

Experimental design is shown in Figure 4.13. Representative histograms of T-bet and GATA-3 expression in iTreg re-stimulated under different conditions, gated on Foxp3⁺ cells. Data are representative of 2 experiments.

4.3. Discussion

4.3.1. Specialization of Treg in Th1- and Th2-mediated inflammation

The suppressive potential of Treg is thought to rely on their ability to accumulate at the site of inflammation to adapt to the inflammatory environment in order to effectively regulate it (Yuan et al., 2007, Kang et al., 2007, Koch et al., 2009). To assess the possibility that Treg may specialize in line with CD4⁺ T cells driving a particular type of inflammation as suggested by other studies, the models of Th1/Th17-driven CNS inflammation and Th2-driven lung inflammation were investigated. The percentage and total cell number of Foxp3⁺ cells were found to be elevated during the recovery phase in EAE as reported previously (McGeachy et al., 2005). Similar observation was made in a new model of rMOG-driven AAI described in Chapter 3 where a significant accumulation of Foxp3⁺ cells was noted specifically within the lung upon antigen airway challenge. Phenotypic analysis revealed that Treg from the inflamed CNS in EAE had an upregulated expression of the Th1 master regulator T-bet and Th1-associated chemokine receptor CXCR3. In contrast, Treg derived from the inflamed lung in AAI had an increased expression of the Th2 master regulator GATA-3, lacked expression of T-bet and displayed decreased levels of CXCR3 compared to the CNS-derived Treg. Importantly, this “specialization” of Treg was only found in those sampled from the sites of inflammation and peripheral Treg displayed the same phenotype as Treg isolated from naïve mice. Foxp3⁺ and effector CD4⁺ cells sourced from EAE CNS and spleen and naïve spleen were also phenotyped by whole genome analysis using Affymetrix and preliminary analysis supports the increased expression of T-bet and CXCR3 in the CNS-derived Treg (data not shown).

The first evidence that Treg might be able to express T-bet came from a study which used a repetitive antigenic stimulation to induce IL-10-secreting CD4⁺ cells (Tr1) in a model of peptide-induced tolerance (Anderson et al., 2006). Gene expression profiling performed in that study indicated T-bet as one of the upregulated genes in the peptide-induced (PI)-Treg which was linked to the suppression of IL-2 transcription thereby contributing to the tolerogenic rather than pathogenic potential

of these cells. Tr1 cells however do not express Foxp3 and hence it was unclear whether T-bet expression within the Treg compartment could also apply to Foxp3⁺ Treg. T-bet expression in Foxp3⁺ cells was reported later in Treg during Th1 type inflammation and, as already mentioned, it was demonstrated to be critical for Treg homeostasis, function and CXCR3-mediated migration to the site of inflammation (Koch et al., 2009). Interestingly, T-bet expression was found to be higher in Foxp3⁻ cells compared to Foxp3⁺ cells in that study which is similar to the observation made in this project, namely that CNS-derived effector T cells have higher T-bet expression compared to Foxp3⁺ cells. This would support the idea that a low T-bet transcription in Treg is needed to switch on gene expression important for the control of Th1 response, whereas high T-bet expression, as found in Th1 cells, is associated with a pathogenic potential.

Pathogenic T-bet expression in Treg was reported in a study involving a lethal infection with *Toxoplasma gondii* (Oldenhove et al., 2009). The highly Th1-polarised environment in this infection model was suggested to disrupt Treg homeostasis, induce high T-bet and IFN- γ expression and contribute to Treg instability and hence inability to suppress CD4⁺ T cell responses. It is unclear however what level of T-bet expression in Treg confers more efficient suppressive phenotype and what level disrupts basic Treg functions.

In addition to the already mentioned expression of the Th2-associated transcription factor IRF4 in Treg (Zheng et al., 2009), an increased GATA-3 expression in Foxp3⁺ cells has been reported at mucosal sites in the gastrointestinal tract and the skin but not in the lung under steady-state conditions (Wohlfert et al., 2011). That study indicated that GATA-3 expression was not required to maintain Treg homeostasis and function at steady state. However, during inflammation it was required for Treg migration to the site of inflammation, for the maintenance of Foxp3 expression stability and for resistance to the acquisition of effector functions (Wohlfert et al., 2011). Interestingly, GATA-3-deficient Treg failed to protect in a model of Th1/Th17-mediated colitis indicating that in this case the site of inflammation rather than the type of immune response dictated Treg phenotype. Utilizing the method of

systemic activation and expansion of Treg with rIL-2 coupled with α -IL-2 antibody that study also demonstrated systemic GATA-3 upregulation in Foxp3⁺ cells and suggested that GATA-3 upregulation observed in Treg related to their activation status and was not a result of adaptation to the cytokine milieu. However, it was also shown that GATA-3 expression can be modified by polarising cytokines *in vitro* which poses a question whether the same modification happens under inflammatory conditions *in vivo*. The results from this project show that Foxp3⁺ cells derived from Th1/Th17 environment express lower levels of GATA-3 versus those derived from Th2 environment when compared to peripheral Treg which would indicate that such modification *in vivo* exists.

It is important to note that although EAE is considered to be mediated by Th1/Th17 cells, this project did not investigate the expression of Th17-associated transcription factors in Treg derived from the CNS due to insufficient cell numbers available for analysis. It has, however, been published that Foxp3⁺ cells can under certain conditions express STAT3, a transcription factor critical for Th17 differentiation which enables them to control pathogenic Th17 responses (Chaudhry et al., 2009).

There is evidence that this phenotypic diversity within the Treg compartment associated with specialization towards the corresponding types of CD4⁺ cell responses which is observed in murine models of inflammation is also relevant to human. Duhon et al. have reported that human peripheral blood contains multiple subsets of Treg characterised by distinct expression of chemokine receptors, transcription factors and cytokine responses (Duhon et al., 2012). Th1-, Th2-, Th17- and Th22-like Treg were differentiated on the basis of chemokine receptor expression including expression of CXCR3 in Th1-like Treg and CCR4 in Th2-like Treg. Moreover, mRNA expression showed higher levels of T-bet in Th1-like Treg as well as elevated ROR- γ t in Th17-like Treg. Increased GATA-3 expression in Th2-like Treg was less conclusive (Duhon et al., 2012). This highlights important differences to the murine model where peripheral Treg do not seem to be specialized, but acquire a distinct phenotype during inflammation specifically within the tissue. It would be of interest to know whether human Treg types distinguished in the

periphery of healthy individuals are more evident during inflammation in the affected tissue itself.

4.3.2. Homing / activation status – tissue-derived versus peripheral Treg

High expression of CD62L in conventional CD4⁺ T cells is associated with a naïve cell phenotype and ability of T cells to recirculate between the blood and the lymph nodes. In Treg, expression of CD62L differentiates several subsets. CD62L^{+/high} Treg have been shown to migrate to lymphoid tissues and suppress activation of T cells within them, whereas CD62L^{-/low} Treg have the ability to enter inflamed tissues and suppress on-going inflammation (Huehn et al., 2004, Stephens et al., 2007). This is consistent with the CD62L⁻ phenotype of tissue-derived Treg observed in this project. Interestingly, both CD62L⁻ and CD62L⁺ Treg populations were isolated from the inflamed lung which may indicate that a subset of lung-derived Treg maintained the capacity to recirculate, whereas the CD62L⁻ Treg from the CNS did not.

Treg isolated from the CNS and the lung tissue had highly upregulated expression of CD44 compared to peripheral steady-state Treg. CD44 is a hyaluronic acid receptor and is commonly used as a T cell activation/memory marker. CD44 is upregulated on Treg upon activation and its high expression positively correlates with their suppressive capacity both *in vitro* and *in vivo* in a model of graft versus host disease (Firan et al., 2006, Bollyky et al., 2007). This suggests that Foxp3⁺ cells derived from the inflamed tissues in both EAE and AAI are activated and may have increased suppressive capacity which could contribute to their ability to effectively resolve inflammation observed in both disease models.

A different cell surface marker that has been also associated with suppressive capacity of Treg is CD73 which was highly elevated on Treg specifically from the inflamed CNS and the lung. CD73 is a surface enzyme that converts local 5'AMP to adenosine which was shown to suppress proliferation and cytokine secretion of Th1 and Th2 cells (Kobie et al., 2006, Deaglio et al., 2007). 5'AMP was suggested to be released by neutrophils or dying cells and hence to contribute to the resolution of

inflammation by dampening excessive damage to the tissue (Kobie et al., 2006). Therefore, higher expression of CD73 on Treg may play an important role in mediating the control and resolution of inflammation in the EAE and AAI models.

4.3.3. Cytokine control of Treg polarisation *in vitro*

Ex vivo CD4⁺ Foxp3⁻ cells isolated from the spleen of non-manipulated mice did not express T-bet and had intermediate levels of GATA-3, which is consistent with previous reports (Lighvani et al., 2001, Zhang et al., 1997). nTreg shared the same phenotype, but their expression of GATA-3 was consistently higher compared to naïve Foxp3⁻ cells. Elevated GATA-3 within the Treg compartment versus the CD4⁺ Foxp3⁻ cell compartment at a steady-state level was reported previously and the most pronounced differences were shown to occur mainly at the site of mucosal barriers (Wohlfert et al., 2011). Interestingly, upregulation of GATA-3 in Treg was demonstrated to occur upon TCR engagement (Wohlfert et al., 2011) and hence could reflect a higher activation status of peripheral Treg, either as a whole population or within a specific Treg subset, compared to Foxp3⁻ cells. Another study which also reported that nTreg at a steady-state level have a higher expression of GATA-3 compared to conventional CD4⁺ cells, suggested that this intrinsic property of nTreg predisposes them towards conversion to Th2 cells contributing to the greater potential plasticity of Treg (Wang et al., 2010).

It has been well established from *in vitro* studies that IL-12 induces differentiation of CD4⁺ cells to Th1 cells (Hsieh et al., 1993, Seder et al., 1993), whereas IL-18 acts as an enhancer (Yoshimoto et al., 1998). IL-12 signalling results in activation of T-bet which controls expression of Th1-characteristic genes such as *ifnγ* and *cxc3* (Kaplan et al., 1996, Szabo et al., 2000). Consistent with these observations, polyclonal stimulation of splenocytes isolated from naïve mice in the presence of IL-12 and IL-18 resulted in upregulation of T-bet in CD4⁺ Foxp3⁻ cells. Relatively high GATA-3 expression in these conditions could be a transient effect of a high activation status. Interestingly, it was demonstrated that IL-12 and IL-18 also upregulated T-bet expression within the Foxp3⁺ cell population. Similar results were obtained by Wei

at al. who showed that Foxp3⁺ cell stimulation in the presence of IL-12 only is sufficient for the induction of T-bet (Wei et al., 2009). Lower expression of T-bet in nTreg versus Th1 cells under polarising conditions is consistent with the *in vivo* data.

A key cytokine driving the differentiation of Th2 cells is IL-4 which activates GATA-3 expression and hence programmes the Th2 cell lineage (Zheng and Flavell, 1997). Stimulation in the presence of IL-4 and blocking antibodies to IL-12 and IFN- γ was found to upregulate expression of GATA-3 in nTreg similarly to conventional CD4⁺ Foxp3⁻ cells indicating that the cytokine driving Th2 cell development also mediates similar transcriptional changes in nTreg. Interestingly, the expression pattern of GATA-3 in nTreg and Foxp3⁻ CD4⁺ cells was very different. Foxp3⁻ CD4⁺ cells uniformly upregulated GATA-3, whereas nTreg seemed to express GATA-3 in a bimodal fashion suggesting that only a subset of Treg was capable of expressing GATA-3 at the same level as conventional CD4⁺ cells. Upregulation of GATA-3 in the presence of IL-4 was also reported by Wohlfert et al and moreover IL-2 was demonstrated in that study to contribute to this (Wohlfert et al., 2011).

It is important to note that although IL-12 and IL-18, and IL-4 were determined to be sufficient for the polarisation of nTreg towards a Th1- or a Th2-like phenotype, respectively, cytokines derived from CD4⁺ Foxp3⁻ cells such as IFN- γ might be implicated as well. However, in order to test this, sorted nTreg would have to be stimulated under polarising conditions and this was found to result in substantial cell death of nTreg over 3-day period.

An interesting observation was made during nTreg expansion under polarising conditions. Whereas, IL-12 and IL-18 were able to induce T-bet and high CXCR3 expression in expanding nTreg, IL-4 was did not promote GATA-3 upregulation. It has been previously demonstrated that Foxp3 can bind to and inhibit GATA-3 expression repressing Th2 cell development and, vice versa, IL-4 was shown to inhibit Foxp3 expression (Wei et al., 2007, Mantel et al., 2007). Therefore, it is possible that upregulation of GATA-3 does not happen in actively proliferating nTreg or it requires additional factors *in vitro*. This would be consistent with the

observed GATA-3 upregulation in nTreg stimulated under Th2 conditions but not in proliferating nTreg during their expansion under the same Th2 conditions.

Although the cytokine production by nTreg under polarising conditions was decided not to be investigated in this project, previous reports have indicated that whereas nTreg stimulated under Th1 polarising conditions can produce small amounts of IFN- γ (Wei et al., 2009), nTreg stimulated in the presence of IL-4 did not acquire an ability to produce Th2-associated cytokines (Wohlfert et al., 2011). This poses interesting questions of the extent to which T-bet and GATA-3 affect the transcriptional machinery in Treg such as the expression of chemokine receptors and cytokines and how this is controlled to maintain a non-pathogenic phenotype.

4.3.4. How do pro-inflammatory cytokines affect *in vitro* generated iTreg?

Phenotypic analysis of steady-state nTreg and *in vitro* TGF- β -induced iTreg revealed substantial differences between the two types of Foxp3⁺ cells. iTreg were found to express intermediate levels of GATA-3, similarly to nTreg. However, they had highly upregulated expression of T-bet and CXCR3. High T-bet expression in iTreg was demonstrated previously and it was shown to be expressed at a similar level compared to IL-12 conditioned Foxp3⁺ cells (O'Connor et al., 2010). iTreg were also characterized by elevated expression of CD44 and CD73, like nTreg. However, they had a higher proportion of CD62L^{high} cells. Due to these differences, it was of interest to know whether the effect of polarising cytokines on the cell phenotype was similar in nTreg and iTreg.

The presence of IL-12 did not affect TGF- β -induced conversion of CD4⁺ CD62L^{high} cells into iTreg *in vitro*, which is consistent with previous studies (Neufert et al., 2007). High expression of T-bet in iTreg also remained unaffected by IL-12. However, as the phenotypic analysis was done on day 5 of induction, and the kinetics of T-bet expression in iTreg was demonstrated to be the highest on day 2 and then decrease (O'Connor et al., 2010), it is possible that IL-12 could exert its effects earlier during the induction process. The presence of IL-4 during the iTreg

generation was observed to inhibit Foxp3 induction in a dose-dependent manner. This is consistent with the already mentioned report that IL-4 has a capacity to suppress Foxp3 expression (Mantel et al., 2007). Interestingly, α -IFN- γ but not α -IL-12, present in Th2-polarising conditions, was able to effectively oppose the inhibitory effects of IL-4 on Foxp3 expression. iTreg are known to produce IFN- γ during their induction (O'Connor et al., 2010) and, hence, this may indicate that its neutralization promotes iTreg conversion under suboptimal conditions when IL-4 is present. Addition of α -IFN- γ on its own did not promote Foxp3 induction and could be explained by highly efficient Foxp3 induction conditions used in this project. An enhancing effect α -IFN- γ on iTreg induction was also reported *in vivo* in tumour-bearing mice (Cao et al., 2009). No GATA-3 upregulation was detected in iTreg generated under Th2 polarising conditions as reported previously (Dardalhon et al., 2008). This could be explained by the reciprocal regulation of Foxp3 and GATA-3 expression (Wei et al., 2007, Mantel et al., 2007) and also by the fact that TGF- β together with IL-4 was demonstrated to result in the generation of a new cell subset called IL-9⁺ IL-10⁺ Foxp3⁻ cells, described as Th9 cells (Dardalhon et al., 2008). However, the production of IL-9 and IL-10 was not investigated in here, hence it is not known whether the cells generated were Th9 cells.

The influence of cytokines on already induced iTreg was also investigated. It was found that iTreg re-stimulated under either Th1 or Th2 polarising conditions rapidly lose Foxp3 expression which was prevented by the addition of TGF- β to the cultures. The instability of Foxp3 in iTreg upon re-stimulation *in vitro* was described previously and is thought to be a result of an incomplete demethylation of the *foxp3* promoter region (Floess et al., 2007, Huehn et al., 2009, Chen et al., 2011). This is in contrast to nTreg which have a fully demethylated *foxp3* promoter region and hence seem to be stable under stimulatory conditions. The presence of IL-12 or IL-4 did not promote upregulation of T-bet or GATA-3 in iTreg, but interestingly, the presence of TGF- β was critical for the maintenance of high T-bet expression and intermediate GATA-3 expression in iTreg upon re-stimulation. This suggests the importance of TGF- β in maintaining not only the Foxp3 stability upon re-stimulation as shown before (Chen et al., 2011) but also the characteristic iTreg phenotype.

The default Th1-like phenotype of iTreg and the inability to modulate it *in vitro* poses a question whether iTreg can be ‘polarised’ *in vivo* in the same way as nTreg. Stock et al. have reported on T helper type 1-like regulatory T cells that developed *in vivo* from CD4⁺ CD25⁻ T cells during a strong Th1 response (Stock et al., 2004). These expressed both T-bet and Foxp3 and effectively inhibited Th2-induced airway hyperactivity. They argued that Th1-like iTreg might counter a Th2-mediated response by secreting IL-10 and IFN- γ . Whether Th2-like iTreg can be generated *in vivo* as well is unknown.

4.4. Concluding synopsis

- Peripheral Foxp3⁺ CD4⁺ cells from naïve mice do not express T-bet and have low/intermediate levels of GATA-3.
- Foxp3⁺ CD4⁺ cells upregulate expression of T-bet and CXCR3 (but not GATA-3) specifically within the inflamed CNS in Th1/Th17-mediated EAE.
- Foxp3⁺ CD4⁺ cells upregulate expression of GATA-3 (but not T-bet and CXCR3) within the inflamed lung in Th2-mediated AAI.
- *In vitro* experiments suggest that IL-12 and IL-18 are responsible for upregulation of T-bet in stimulated nTreg, whereas IL-4 is responsible for upregulation of GATA-3.
- TGF- β -induced iTreg have a substantially different phenotype to steady-state nTreg and display a default Th1-like phenotype with high T-bet and CXCR3 expression.
- The phenotype of iTreg cannot be modified by polarising cytokines during their generation *in vitro* which seem to mainly affect the induction of Foxp3 expression.
- iTreg re-stimulated under Th1 or Th2 polarising conditions *in vitro* do not upregulate T-bet or GATA-3, respectively.
- TGF- β is critical to the maintenance of Foxp3 expression and Th1-like iTreg phenotype upon re-stimulation.

The functional consequences of Treg polarisation and the importance of Treg phenotype versus antigen reactivity are discussed in Chapter 5.

5 Functional consequences of Treg phenotype / polarisation

5.1. Introduction

The importance of Treg phenotype for their effective suppressive capacity was suggested by several studies. T-bet expression was demonstrated to be critical for Treg-mediated inhibition of CD4⁺ T cell responses in a model of Th1-mediated infection by affecting Treg function, homeostasis and migration (Koch et al., 2009). Expression of Th2-associated IRF-4 was shown to be important for the control of Th2 responses (Zheng et al., 2009). However, it has been never addressed whether polarised Treg phenotype makes them ineffective at mediating suppression driven by non-corresponding CD4⁺ T cells, or whether the Treg phenotype is plastic and can adapt to a different inflammatory environment.

The marked difference between nTreg and *in vitro* generated iTreg that has been noted and described in Chapter 4 is that the default Th1-like iTreg phenotype, in contrast to nTreg, could not be skewed towards being more Th1-like (with higher T-bet expression) or Th2-like (with upregulated GATA-3) by polarising cytokines, unless iTreg stability was compromised. Whether this observation has functional consequences needs to be investigated. Interestingly, it has been demonstrated that iTreg induced *in vivo* during a strong Th1-response can effectively suppress Th2-driven inflammation (Stock et al., 2004) suggesting that iTreg, in contrast to nTreg, may not need to adapt to the inflammatory environment in order to effectively regulate it.

It is still unclear whether antigen reactivity is critical to iTreg-mediated suppression. Multiple studies on autoimmune disease models such as diabetes, gastritis and EAE have demonstrated protective effects of antigen-reactive iTreg only (Tang et al., 2004, Huter et al., 2008, Zhang et al., 2010), however some showed suppressive potential of polyclonal iTreg as well (Selvaraj and Geiger, 2008). Interestingly, the majority of investigations into Th2-mediated sensitization models of asthma show

inhibitory effects of polyclonal iTreg (Chen et al., 2003, Xu et al., 2012), however no comparison to antigen-reactive iTreg has been made.

5.1.1. Aims

The aims of the experiments described in this chapter were to:

- 1) Assess the importance of polarised nTreg phenotype *in vitro* and *in vivo* in models of Th1/Th17-mediated EAE and Th2-mediated AAI.
- 2) Establish the suppressive capacity of T-bet⁺ CXCR3⁺ iTreg towards pre-activated polarised CD4⁺ T cells *in vitro*.
- 3) Investigate the prophylactic and therapeutic potential of iTreg in EAE and AAI and determine how important iTreg antigen reactivity is.
- 4) Assess whether iTreg are able to mediate bystander suppression in a model of AAI.

5.1.2. Approach

To investigate the effect of a polarised nTreg phenotype on their suppressive capacity *in vitro*, a standard suppression assay previously described by the Shevach group was used (Thornton and Shevach, 1998, Thornton and Shevach, 2000). It consisted of an unchanged number of CD4⁺ conventional T responder cells (Tresp), either naïve or pre-activated, and APC incubated with serially diluted nTreg in the presence of a TCR stimulus. For functional assessment *in vivo*, T-bet⁺ CXCR3^{high} Treg were isolated from the CNS of EAE mice and transferred into an AAI model prior to allergen challenge. As Treg derived from the inflamed CNS in EAE were demonstrated to be enriched for MOG-reactive Treg (O'Connor et al., 2007, Korn et al., 2007), and rMOG-induced AAI is driven by MOG-reactive CD4⁺ cells, this model was used to distinguish between the effects of polarised Treg phenotype and their antigen reactivity. To isolate and sort for nTreg, Foxp3-GFP mice were used in these experiments.

For the investigation of functional consequences of the default Th1-like iTreg phenotype, *in vitro* suppression assays and models of EAE and AAI were utilized. In EAE, the ability of iTreg to inhibit disease induction at the priming stage and to suppress on-going CNS inflammation was investigated. In AAI, the prophylactic potential of iTreg to suppress pre-activated polarised Th2 cells prior to the establishment of lung inflammation was tested. The effect of iTreg on on-going lung inflammation could not be tested due to the acute nature of rMOG-induced AAI. Antigen-reactive and polyclonal iTreg, generated *in vitro* from 2D2xFoxp3.LuciDTR4 and Foxp3.LuciDTR4 mice respectively, were compared.

5.2. Results

5.2.1. Regulation by nTreg

5.2.1.1. nTreg suppress naïve CD4⁺ T cells *in vitro*

To verify the effective use of an *in vitro* suppression assay in this project and establish the baseline efficiency of nTreg suppression, unchanged numbers of naïve conventional CD4⁺ T responder cells (Tresp) were incubated with serially diluted freshly isolated nTreg in the presence of irradiated APC and were stimulated with α -CD3 (Figure 5.1A). Proliferation of Tresp was measured on day 4 by ³H thymidine incorporation. nTreg effectively suppressed polyclonal proliferation of naïve CD4⁺ cells and suppression of more than 50% of proliferation was achieved until the nTreg to Tresp ratio reached 1:16 (Figure 5.1B).

5.2.1.2. Suppression by nTreg expanded under polarising conditions

In order to investigate the functional consequences of polarised Treg phenotype, nTreg expanded under different polarising conditions were tested for their suppressive capacity *in vitro*. Polyclonal nTreg expanded *in vitro* under neutral, Th1 or Th2 polarising conditions were first incubated with naïve Tresp in the presence of APC and were stimulated with α -CD3 (Figure 5.2A). All nTreg populations achieved 50% suppression at approximately 1:16 ratio (Figure 5.2B&C).

Neutral, Th1- and Th2-conditioned nTreg were then tested for their ability to suppress polyclonal proliferation of pre-activated Th1 and Th2 cells. All nTreg populations suppressed Th1 and Th2 cells with similar efficacy (Figure 5.3A&B). Higher nTreg to Tresp ratios were required to achieve 50% suppression of pre-activated T cells compared to naïve CD4⁺ cells. Moreover, Th1 cells were found to be more resistant to suppression than Th2 cells even though Th2 cells had a higher proliferation rate (data not shown).

5.2.1.3. T-bet⁺ CNS-derived Treg do not suppress rMOG-induced AAI

To assess the functional importance of the polarised Treg phenotype (T-bet⁺ CXCR3^{high}) that was observed in Th1/Th17-mediated EAE, Treg were isolated from the inflamed CNS in EAE and tested in the AAI model by transfer (Figure 5.4A). To ensure sufficient cell numbers obtained from the CNS, cells were harvested 3 days after Foxp3-GFP mice reached peak of disease and not during resolution (Figure 5.4B). These CNS-derived Treg had similar expression levels of T-bet and CXCR3 as those isolated during resolution of EAE (Figure 4.1D). Treg from the CNS and the control Treg from naïve spleen were FACS sorted for GFP⁺CD4⁺ cells and over 99% purity was achieved (Figure 5.4C). All transferred cell populations were traceable.

Lower than average inflammatory response was achieved in this experiment. Total cell numbers in BAL were unaffected by CNS-derived Treg (Figure 5.4D) as were the percentages of BAL eosinophils (Figure 5.4E). Lung infiltration scores in the peri-vascular and peri-bronchiolar areas were similar in all groups (Figure 5.5A&B), and the percentages of goblet cells in small and large airways remained unchanged in the group that received CNS-Treg (Figure 5.5A&C). The percentages of goblet cells in large airways of mice that received naïve nTreg were significantly increased compared to rMOG only control (Figure 5.5C).

The total cell number in the lung seemed lower in the CNS-Treg group however it was not significantly different (Figure 5.6A). The total cell numbers in medLN and spleen were unaffected (Figure 5.6B). The percentages of CD4⁺ cells in the lung,

medLN and spleen were similar in all groups (Figure 5.6C). Percentages and total numbers of transferred 2D2 Th2 cells in the lung, medLN and spleen were unchanged by CNS-Treg or naïve Treg transfer (Figure 5.6B). Percentages and absolute numbers of total Foxp3⁺ cells were increased in the lung irrespective of CNS or naïve Treg transfer in all rMOG challenged groups (data not shown) which is characteristic of lung inflammation as described in Chapter 3. No transferred Treg were detectable in any of the groups (data not shown).

The lack of a protective effect of CNS-derived Treg on the lung inflammation in AAI could suggest that T-bet⁺ CXCR3^{high} Treg were unable to effectively suppress pre-activated Th2 cells. Due to the complicated design of this experiment it was decided to take an alternative approach to verify this result. It involved an induction of Th1-mediated AAI by Th1-polarised 2D2 cells and an assessment of Treg phenotype in this model. If the Treg were found to have a Th1-like phenotype, they would then be transferred into Th2-mediated AAI model to test their suppressive capacity.

5.2.1.4. Treg in Th1-mediated AAI do not acquire expression of T-bet

A model of Th1-mediated AAI was previously described involving an adoptive transfer of Th1-polarised OVA₃₂₃₋₃₃₉-reactive CD4⁺ cells followed by OVA airway challenge (Hansen et al., 1999). It was shown in that model that Th1 cells were capable of inducing severe lung inflammation characterized by predominant lymphocyte infiltrates in the lung instead of eosinophils, and a smaller degree of goblet cell hyperplasia compared to the Th2-mediated AAI. Th2- and Th1-mediated AAI were compared here by polarisation of 2D2 cells towards either a Th2 or a Th1 phenotype for transfer into WT hosts followed by airway challenges with rMOG (Figure 5.7A). Total cell numbers in BAL were increased after transfer of either Th1 or Th2 cells, and rMOG challenge (Figure 5.7B). Th2 cells induced BAL eosinophilia as described previously, whereas the transfer of Th1 cells resulted in very few eosinophils and increased percentages of neutrophils and lymphocytes (Figure 5.7C). Lung infiltration scores in rMOG challenged mice that received Th1 or Th2 cells were increased (Figure 5.7D). The percentages of goblet cells in small

and large airways were increased in rMOG-challenged mice that received Th2 cells, and negligible in mice that received Th1 cells (Figure 5.7E) which is consistent with previously published observations (Hansen et al., 1999).

The percentages and total numbers of transferred Th1 and Th2 2D2 cells in the lung were increased by rMOG challenge (data not shown, Figure 5.8A) as were the total numbers of Foxp3⁺ cells (Figure 5.8B). The stability of Th1 or Th2 phenotype was confirmed by overnight re-stimulation of cells isolated from the lung with MOG₃₅₋₅₅ followed by intracellular staining for cytokines. Approximately 8-10% of Th2 2D2 cells produced IL-13 and fewer than 3% produced IFN- γ , whereas 20-25% of Th1 2D2 cells secreted IFN- γ and fewer than 2% produced IL-13 (Figure 5.8C). Staining for transcription factors revealed that although both lung-derived Th2 2D2 cells and Foxp3⁺ cells from the Th2-mediated AAI had an upregulated GATA-3 expression, Th1 2D2 cells and the corresponding Foxp3⁺ cells did not express T-bet, as was expected (Figure 5.8D). Lack of T-bet expression by Foxp3⁺ cells in Th1-mediated AAI meant that this model could not be used for testing the importance of the Treg phenotype in different inflammatory contexts.

5.2.2. Regulation by iTreg

In addition to nTreg which are present in the periphery at a relatively low frequency, *in vitro* induced Treg (iTreg) provide an easy means of generating robust numbers of cells capable of effectively suppressing pathogenic T cell responses. Moreover, as iTreg display a Th1-like phenotype, this provides an opportunity to test whether high expression of T-bet and CXCR3 is implicated in the failure of CNS-derived Treg to suppress Th2-mediated AAI.

5.2.2.1. 2D2 iTreg suppress proliferation of naïve 2D2 CD4⁺ T cells *in vitro* more effectively than polyclonal iTreg

The importance of iTreg antigen reactivity for effective suppression of CD4⁺ cells was first tested *in vitro*. 2D2 iTreg and polyclonal (poly) iTreg were induced *in vitro* in the presence of TGF- β and IL-2 as described previously. The average Foxp3

induction efficiency from both 2D2 and WT mice was >85-95% of total CD4⁺ cells on day 5. iTreg were sorted for GFP⁺ cells using FACS prior to culture set up. Unchanged numbers of naïve 2D2 CD4⁺ cells (Tresp) were incubated with serially diluted 2D2 iTreg or poly iTreg in the presence of irradiated APC and were stimulated with MOG₃₅₋₅₅ (Figure 5.9A). 2D2 iTreg were found to suppress antigen-driven proliferation of naïve 2D2 CD4⁺ cells more effectively than poly iTreg (Figure 5.9B). 50% suppression was achievable using 2D2 iTreg at iTreg to Tresp ratios of 1:32 and above whereas this was lost with poly iTreg below a ratio of 1:4 (Figure 5.9B).

5.2.2.2. 2D2 iTreg proliferate *in vivo* in response to antigen but partially lose Foxp3 expression

To determine the potential of 2D2 iTreg to suppress naïve CD4⁺ T cell activation / proliferation *in vivo*, their ability to migrate and proliferate in the dLN in response to immunization was first assessed. CD90.1⁺ CD90.2⁺ 2D2 iTreg were transferred into WT mice without prior FACS sort for GFP⁺ cells as the 2D2 mouse used was not on a Foxp3-GFP reporter background. This was followed by immunization of WT mice with MOG₃₅₋₅₅ in CFA (Figure 5.10A). Unimmunized mice were used as a control. On day 4 post-immunization, dLN and spleen were harvested. On the day of transfer, the iTreg population was ~85% Foxp3⁺ and the cells expressed high levels of Ki-67, known to be expressed by actively proliferating cells (Figure 5.10B).

Four days after immunization, the total number of 2D2 iTreg in the dLN was significantly higher compared to the unimmunized control (Figure 5.10C). Ki-67 staining revealed that ~60% of total 2D2 iTreg (Figure 5.10C) and ~50% of Foxp3⁺ iTreg (data not shown) were actively proliferating in response to antigen *in vivo*. This was associated with the loss of Foxp3 expression observed in ~50% of iTreg from ~85% Foxp3⁺ iTreg on the day of transfer (Figure 5.10C). However, since the transferred 2D2 iTreg population was unsorted, it is possible that the Foxp3⁻ cells expanded more than Foxp3⁺ cells upon immunization and this could partially account for the observed decrease in Foxp3 expression. 2D2 iTreg that did not see an antigen *in vivo*, stopped proliferating but retained their expression of Foxp3 (Figure 5.10C).

A similar pattern of results was seen in the spleen with the exception that 2D2 iTreg in the presence of an antigen retained higher levels of Foxp3 ranging from 40-50% (Figure 5.10D). These results suggest that 2D2 iTreg are able to migrate to dLN and proliferate in response to their cognate antigen *in vivo*.

5.2.2.3. 2D2 iTreg limit the survival of naïve 2D2 cells and effector cytokine production *in vivo*

To test the *in vivo* suppressive potential of 2D2 iTreg, these were generated from a 2D2 mouse and transferred into WT host together with traceable naïve 2D2 CD4⁺ cells (Tresp) at a 1 to 1 ratio followed by immunization with MOG₃₅₋₅₅ in CFA (Figure 5.11A). Mice that received Tresp or 2D2 iTreg only and unimmunized mice that received both Tresp and 2D2 iTreg were used as controls. Analysis of dLN 4 days after immunization showed that the total number of Tresp was significantly reduced upon co-transfer of 2D2 iTreg (Figure 5.11B). Interestingly, the vast majority of Tresp were still Ki-67⁺ (Figure 5.11B) which indicates that 2D2 iTreg did not inhibit Tresp proliferation but rather limited their survival *in vivo*. The same pattern of results was obtained in a separate experiment where Tresp were labelled with CFSE proliferation dye (data not shown). Similar reduction in the total numbers of Tresp was observed in the spleen, however smaller suppression of Tresp proliferation was noted (Figure 5.11C) and this was most likely due to the lower percentage of iTreg present in the spleen versus the dLN (data not shown). Although the percentages of 2D2 iTreg in dLN were similar in the immunized groups with or without Tresp, the total numbers of iTreg were higher when Tresp were co-transferred (data not shown) which suggests that 2D2 iTreg migrated to the dLN more effectively in response to inflammatory reaction driven by Tresp. The loss of Foxp3 expression by 2D2 iTreg (~70% in dLN and ~40-50% in the spleen) was similar irrespective of Tresp co-transfer (data not shown).

To investigate the effects of 2D2 iTreg on effector cytokines produced in response to immunization, cells isolated from the dLN and spleen were re-stimulated with increasing concentrations of MOG₃₅₋₅₅ for 3 days followed by cytokine assay. 2D2 iTreg were found to significantly reduce the production of IFN- γ , IL-17A and GM-

CSF in dLN (Figure 5.12A) and of IL-17A in the spleen (Figure 5.12B). This reduction in effector cytokines is most likely due to the reduced percentages of Tresp when co-transferred with 2D2 iTreg (data not shown).

5.2.2.4. 2D2 iTreg increase Foxp3 expression within transferred 2D2 cells

In addition to limiting the survival of Tresp *in vivo* and the levels of effector cytokines, 2D2 iTreg were also found to increase the percentages and total numbers of Foxp3⁺ Tresp within the dLN and spleen upon immunization (Figure 5.13A, B&C). The percentage of Foxp3⁺ cells within 2D2 naïve CD4⁺ cells prior to transfer varied between 2-4% (data not shown) and it was found to increase to ~ 20-25% in unimmunized control (Figure 5.13A&B). This could be due to better survival of Foxp3⁺ cells versus Foxp3⁻ cells in the absence of an antigen *in vivo*. The percentage of Foxp3⁺ Tresp decreased to less than 1% upon immunization (Figure 5.13B) which was due to the rapid expansion of Foxp3⁻ cells, as the total numbers of Foxp3⁺ Tresp remained unaffected in the unimmunized control and in the Tresp only group (Figure 5.13C). 2D2 iTreg were observed to significantly increase the percentages of Foxp3⁺ Tresp to ~11-12% in dLN and ~20% in the spleen, and increase the total numbers of Foxp3⁺ Tresp (Figure 5.13A, B&C). Whether this increase is due to the iTreg-mediated expansion of pre-existing Foxp3⁺ cells within the transferred Tresp in the presence of an antigen, or true Foxp3 induction in naïve 2D2 upon activation needs further investigation.

5.2.2.5. 2D2 iTreg but not polyclonal iTreg suppress induction of EAE

Previously published reports demonstrated that antigen-reactive iTreg but not polyclonal iTreg are capable of suppressing EAE induction in a model of PLP₁₃₉₋₁₅₁ driven active EAE (Zhang et al., 2010). They suggested that iTreg suppress induction of EAE in an antigen-dependent fashion similarly to nTreg. Contrasting results were shown by an earlier paper which reported that in a model of MOG₃₅₋₅₅-driven EAE polyclonal iTreg are effective at suppressing disease severity (Selvaraj and Geiger,

2008). However, no comparison was made between the suppressive potential of antigen-reactive and polyclonal iTreg in that study. Results from the experiments described in this Chapter suggest that 2D2 iTreg suppress naïve cell activation / proliferation more efficiently than polyclonal iTreg, and moreover they expand *in vivo* in response to the antigen and effectively inhibit CD4⁺ T cell responses. In order to establish whether these results reflect what happens during EAE induction, traceable 2D2 iTreg and polyclonal iTreg were transferred into WT mice prior to immunization with MOG₃₅₋₅₅ in CFA for the induction of EAE (Figure 5.14A). PBS-treated mice were used as a control.

It was found that 2D2 iTreg but not polyclonal iTreg suppressed EAE induction. The lower dose of 2D2 iTreg, namely 2×10^5 cells was able to significantly reduce disease severity whereas the same dose of polyclonal iTreg had no effect on a disease course (Figure 5.14B). The mice that received a higher dose of 2D2 iTreg, namely 1×10^6 cells remained 100% disease-free (Figure 5.14B). Total cell numbers and numbers of CD4⁺ cells were significantly reduced in the CNS of mice that received 1×10^6 of 2D2 iTreg (Figure 5.14C&D). However, the cellularity and total numbers of CD4⁺ cells within dLN were significantly higher in these mice and in mice that received a lower dose of 2D2 iTreg compared to the groups treated with poly iTreg or PBS (Figure 5.14C&D). In the spleen, the total cellularity and total numbers of CD4⁺ cells were significantly reduced in sick mice treated with PBS or poly iTreg which is consistent with the observed leukopenia in EAE (Figure 5.14C&D). This was prevented by 2D2 iTreg treatment (Figure 5.14C&D). These observations suggest that the protective effect of 2D2 iTreg could be due to reduced mobilization of cells, including effector CD4⁺ cells, from the dLN (and the spleen) into the CNS.

To test whether 2D2 iTreg affected effector functions of CD4⁺ cells, the cells from the CNS, dLN and spleen were re-stimulated overnight with MOG₃₅₋₅₅ followed by intracellular cytokine staining. Within the CNS, mice that received 1×10^6 2D2 iTreg had significantly lower percentages of IFN- γ ⁺, TNF- α ⁺ and GM-CSF⁺ CD4⁺ cells (Figure 5.15A&B). Interestingly, the percentages of IL-17A⁺ CD4⁺ cells were unaffected by 2D2 iTreg. The percentages of IFN- γ ⁺, TNF- α ⁺, GM-CSF⁺ and IL-

17A⁺ CD4⁺ cells were reduced in dLN, and remained unchanged in the spleen (Figure 5.16A&B).

5.2.2.6. 2D2 iTreg accumulate in dLN when transferred prior to EAE induction

iTreg transferred into WT host were traceable which enabled identification of the potential site at which they accumulated and exerted their suppressive effect. Numbers of poly iTreg and 2D2 iTreg at both doses were very low or negligible in the CNS itself (Figure 5.17A). This suggests that 2D2 iTreg either migrated to the CNS at an earlier time point and quickly died off after effector CD4⁺ cells had been effectively suppressed, or that the CNS was not the main site at which they exerted their suppressive function. Analysis of dLN and spleen revealed that 2D2 iTreg numbers in the dLN were elevated compared to poly iTreg numbers, and moreover they were higher compared to their numbers in the spleen (Figure 5.17A). This confirms antigen-driven migration/accumulation of 2D2 iTreg in the dLN as previously shown 4 days post-immunization (Figure 5.10). iTreg stability could not be assessed due to the low cell numbers which did not allow for reliable analysis of Foxp3 expression.

When Foxp3 expression by total CD4⁺ cells was compared, it was found that the percentages of Foxp3⁺ CD4⁺ cells in the CNS were unaffected by 2D2 iTreg treatment, but their total numbers were significantly reduced in the group that received 1x10⁶ 2D2 iTreg (Figure 5.17B). This can be explained by the fact that the animals were disease-free and had no inflammation in the CNS that would drive the accumulation of Foxp3⁺ cells. The mice that received 1x10⁶ 2D2 iTreg had, however, higher percentages and total numbers of Foxp3⁺ CD4⁺ cells within dLN and spleen compared to PBS and poly iTreg-treated groups (Figure 5.17A&B). Whereas the total numbers of Foxp3⁺ cells within spleen of 2D2 iTreg-treated mice were within normal range found in naïve mice, the numbers within dLN were elevated (Figure 5.17B). The elevated numbers of Foxp3⁺ cells in dLN corresponded to the increased total cellularity of dLN.

5.2.2.7. iTreg suppress proliferation and cytokine production by activated effector T cells *in vitro* in an antigen-dependent manner

Having established that 2D2 iTreg effectively suppress naïve cells *in vitro* and *in vivo*, it was decided to determine their suppressive capacity against activated and polarised cells. A standard suppression assay was used as described previously and pre-activated polarised cells were obtained by stimulation of 2D2 splenocytes under Th1, Th2, Th17 or GM-CSF-polarising conditions for 3 days *in vitro* followed by 3 to 4 day rest in IL-2. Polarised cells were sorted for CD4⁺ cells (Tresp) prior to co-culture with iTreg and irradiated APC in the presence of MOG₃₅₋₅₅ (Figure 5.18A). 2D2 iTreg were able to suppress proliferation of all polarised Tresp and they were more effective than poly iTreg (Figure 5.18B). The 50% suppression of proliferation of activated cells mediated by 2D2 iTreg was effective to an iTreg to Tresp of 1:16 for Th1 cells, 1:2 for Th2 cells, 1:8 for Th17 cells and 1:2 for GM-CSF⁺ cells. These ratios were variable among experiments, however, they were always the highest for GM-CSF⁺ cells suggesting that these cells are the most resistant to iTreg-mediated suppression.

In terms of effector functions of polarised Tresp, 2D2 iTreg effectively inhibited IFN- γ production by Th1 cells, IL-4 and IL-13 by Th2 cells and IL-17A by Th17 cells (Figure 5.19). Interestingly, although polyclonal iTreg were found to suppress some IFN- γ secretion they failed to suppress Th2-associated cytokines and IL-17A. GM-CSF secretion by GM-CSF polarised cells was not suppressed efficiently by either 2D2 iTreg or poly iTreg (Figure 5.20A). However, it was partially inhibited by 2D2 iTreg when GM-CSF was produced by Th1 or Th2 cells (Figure 5.20B).

5.2.2.8. 2D2 iTreg accumulate in CNS during EAE but are unable to suppress on-going inflammation

In order to determine whether iTreg would be able to suppress activated CD4⁺ cells *in vivo* in a disease context, 1×10^6 2D2 iTreg or poly iTreg were transferred into WT mice post EAE induction when animals showed first clinical signs of disease (Figure 5.21A). PBS-treated mice were used as a control. 2D2 iTreg and polyclonal iTreg were induced from 2D2xFoxp3.LuciDTR4 and Foxp3.LuciDTR4 mice, respectively,

and the purity of Foxp3⁺ cells prior to transfer was >99% (Figure 5.21B). 2D2 iTreg and poly iTreg were not found to suppress disease severity or lead to an earlier disease resolution (Figure 5.21C). Tissues were harvested close to the peak of EAE, on day 13, and as the animals were recovering, on day 23.

The total cell numbers and numbers of CD4⁺ cells in the CNS and dLN on day 13 were unaffected by 2D2 iTreg or poly iTreg transfer (data not shown). Similarly, the production of pro-inflammatory cytokines within the CNS and dLN was unchanged which corresponds with the unaffected disease course upon iTreg transfer (data not shown). 2D2 iTreg were detectable in the CNS and their percentage and total number seemed higher compared to poly iTreg, however this difference was not significant (Figure 5.21A). The total number of 2D2 iTreg was 70-fold higher in the dLN (and in the spleen) compared to the CNS, and their numbers were not significantly different to poly iTreg numbers. On day 23, the disease readouts such as total cell numbers in the CNS and dLN, and cytokine production were similar in all three groups (data not shown). Interestingly, the percentage of 2D2 iTreg was significantly increased specifically within the CNS compared to the percentage of poly iTreg, and the total cell number of 2D2 iTreg was ~10-fold higher compared to day 13 (Figure 5.22A&B). The total number of 2D2 iTreg in dLN was 60-fold lower compared to day 13 and comparable to the number of poly iTreg (Figure 5.22A).

These results suggest that 2D2 iTreg accumulated specifically within the CNS as the disease progressed, however they were unable to suppress on-going inflammation. In terms of iTreg stability, ~60% of 2D2 iTreg from the CNS and ~30% in the dLN and spleen had lost their expression of Foxp3 (Figure 5.22C). Polyclonal iTreg stability within CNS could not be analysed due to low cell numbers. Polyclonal iTreg from the dLN and spleen had a similar percentage loss of Foxp3 as 2D2 iTreg (Figure 5.22C) suggesting that the loss of Foxp3 expression was due to inflammation and not due to antigen-driven re-activation of iTreg *in vivo*.

In conclusion, iTreg were demonstrated to be capable of suppressing Th1/Th17-mediated EAE induction in an antigen-dependent manner but not an on-going

disease. Whether this is due to their inability to suppress on-going inflammation itself or an inability to control activated Th1 and Th17 cells needs to be investigated. It was next decided to test whether iTreg would be able to inhibit pre-activated Th2 cells in a model of rMOG-induced AAI and whether this would be antigen-dependent was well.

5.2.2.9. 2D2 iTreg migrate to the lung and medLN upon rMOG airway challenge

Polyclonal iTreg can suppress the Th2-driven sensitization model of AAI when transferred prior to antigen challenge or after the first challenge (Xu et al., 2012) suggesting that iTreg can effectively control CD4⁺ cell responses once the memory Th2 cells have developed but before the inflammation is established. It is not known, however, whether antigen-reactive iTreg would be more effective or whether iTreg will be able to suppress pre-activated Th2 cells *in vivo*. In order to answer these questions, it had to be first determined whether 2D2 iTreg could migrate specifically into the medLN and the lung upon airway challenge with rMOG. WT hosts received 2x10⁶ of traceable 2D2 iTreg followed by a single intratracheal injection with rMOG or PBS as a control (Figure 5.23A). The tissues were harvested two days after the challenge. The percentages and total cell numbers of 2D2 iTreg were increased specifically within the lung and medLN upon rMOG challenge compared to PBS challenged mice, whereas in the spleen they were similar (Figure 5.23B&C). Nevertheless, the total number of 2D2 iTreg in the challenged spleen itself was higher compared to the lung and medLN suggesting that more antigen challenges and time is needed for iTreg to be mobilized into the lung.

5.2.2.10. iTreg suppress rMOG-induced AAI driven by pre-activated Th2 cells in antigen-dependent fashion

In order to test the potential of iTreg to suppress pre-activated Th2 cells *in vivo*, WT hosts received 5x10⁶ 2D2 iTreg or polyclonal iTreg followed by AAI induction via an adoptive transfer of activated Th2 2D2 cells and rMOG airway challenges (Figure 5.24A). All cell populations were traceable. Although no significant changes in the

total numbers of cells in BAL were detected (Figure 5.24B), 2D2 iTreg but not polyclonal iTreg suppressed the percentage and total number of BAL eosinophils (Figure 5.24C&D). The total numbers of macrophages were similar in all groups (Figure 5.24E). Lung infiltration scores were reduced upon 2D2 iTreg treatment (Figure 5.25A&B). Moreover, in mice that received 2D2 iTreg (but not polyclonal iTreg) goblet cells were not evident in either the small or large airways (Figure 5.25A&B).

The total cell numbers and percentages of CD4⁺ cells in the lung and medLN between groups did not differ significantly (Figure 5.26A). However, the percentages and total numbers of transferred Th2 2D2 cells were significantly lower within the lung of 2D2 iTreg-treated mice (Figure 5.26B). The total numbers of Th2 2D2 cells were also significantly lower in the medLN in the same group (Figure 5.26B). In the spleen, the percentages of Th2 2D2 cells were significantly lower in all the groups when compared to the PBS control. However, the absolute numbers were unchanged (Figure 5.26B).

To assess whether effector function of the transferred Th2 2D2 cells was affected, cells from the lung, medLN and the spleen were re-stimulated overnight in the presence or absence of MOG₃₅₋₅₅ followed by intracellular cytokine staining. The percentage of IL-13⁺ Th2 2D2 cells in the lung was reduced from 10% in the PBS-treated group to 4% in the 2D2 iTreg-treated group (Figure 5.27A). The lungs from the group treated with polyclonal iTreg were not analysed in this experiment. The percentages of IL-13⁺ Th2 2D2 cells within medLN were significantly lower in both 2D2 iTreg- and poly iTreg-treated groups and the percentage of IL-5⁺ 2D2 cells was reduced upon 2D2 iTreg transfer only (Figure 5.27B). Similar pattern of results was observed in the spleen (Figure 5.27C).

Interestingly, it was observed that only in mice that received 2D2 iTreg, the percentages of IFN- γ ⁺ 2D2 cells were significantly increased from 1-2% to 7% in medLN and from 2-4% to 15% in the spleen (Figure 5.27B&C). The absolute numbers of IFN- γ ⁺ 2D2 cells cannot be reliably calculated after overnight re-

stimulation. Hence, it is not known whether this increase in the percentage corresponds to elevated total numbers of IFN- γ^+ 2D2 cells or is a result of a decreased expansion of IL-13 and IL-5-producing 2D2 cells in the 2D2 iTreg-treated group.

5.2.2.11. Highest iTreg to Th2 cell ratio is found in the lung

No significant differences in the percentages or total numbers of 2D2 iTreg and poly iTreg within the lung were found although 2D2 iTreg seemed to be elevated (Figure 5.28A). Interestingly, the percentage of 2D2 iTreg was significantly higher in the medLN compared to polyclonal iTreg (Figure 5.28A). The percentages and numbers of 2D2 iTreg and polyclonal iTreg in the spleen were similar (Figure 5.28A). The ratio of 2D2 iTreg to Th2 2D2 cells was the highest in the lung and was ~2 compared to below 1 in the medLN and the spleen (Figure 5.28B). Moreover, it was significantly higher than the ratio of polyclonal iTreg to Th2 2D2 cells in the lung (Figure 5.28B).

Approximately ~65% of 2D2 iTreg and ~ 50% of polyclonal iTreg retained their Foxp3 expression within the lung compared to ~80% in the medLN and spleen in both groups (Figure 5.28C). This suggests that Foxp3 loss is due to inflammation rather than antigen re-stimulation of iTreg *in vivo*.

5.2.2.12. Induction of Foxp3 expression in Th2 2D2 cells by 2D2 iTreg

When Th2 2D2 cells were analysed for Foxp3 expression, it was observed that the frequency of Foxp3 $^+$ cells upon rMOG challenge increased to 10% specifically within the lung compared to less than 1% in the PBS-challenged group (Figure 5.29A&B). 2D2 iTreg but not polyclonal iTreg were found to promote this effect in the lung in terms of the frequency of Foxp3 $^+$ Th2 2D2 cells but not the total cell numbers (Figure 5.29A&B). Interestingly, the percentages but not total cell numbers of Foxp3 $^+$ Th2 2D2 cells seemed higher in the medLN and spleen upon 2D2 iTreg but not polyclonal iTreg treatment as well. In order to determine whether this

increase was due to the iTreg-mediated expansion of Foxp3⁺ cells within transferred Th2 2D2 cell population in the presence of an antigen or true Foxp3 induction in Th2 2D2 cells, it was decided to deplete 2D2 Th2 cells of resident Foxp3⁺ cells prior to transfer for the induction of AAI (Figure 5.30A).

Splenocytes from 2D2xFoxp3.LuciDTR4 mouse were used to polarise cells towards Th2 phenotype. Following stimulation under Th2 polarising conditions the frequency of Foxp3⁺ cells within Th2 2D2 cells was 0.8% and was reduced to 0.12% by sorting out GFP⁺ cells using FACS (Figure 5.30B). It was found that the percentage and total numbers of Foxp3⁺ Th2 2D2 cells were significantly increased in the medLN and the spleen in the group that received 2D2 iTreg but not polyclonal iTreg (Figure 5.30C). The percentage of Foxp3⁺ Th2 2D2 cells increased from ~1.5-2% in the rMOG only and polyclonal iTreg groups to ~12% in the 2D2 iTreg group within the medLN and ~5.5% in the spleen. Interestingly, in mice that were challenged with rMOG and did not receive any iTreg, the percentages and numbers of Th2 2D2 Foxp3⁺ cells seemed higher compared to the PBS control. The lung could not be analyzed due to insufficient cell numbers obtained. These results indicate that iTreg are capable of inducing Foxp3 expression within pre-activated Th2 cells in an antigen-dependent fashion and this could contribute to their protective effect seen in AAI.

5.2.2.13. 2D2 iTreg do not suppress AAI driven by a different antigen

Treg have been suggested to be capable of mediating suppression using a variety of mechanisms one of them being a bystander suppression, whereby they can inhibit CD4⁺ cells reactive to a different antigen via, for example, local production of anti-inflammatory IL-10 and TGF- β (Groux, 2003, Roncarolo et al., 2006). This phenomenon has been reported to occur *in vivo* in a model of arthritis (Zonneveld-Huijssoon et al., 2011). However, in EAE it has been shown that PLP-reactive iTreg are unable to suppress EAE induced by a different antigen to the one that iTreg are reactive to even when iTreg cognate antigen is present (Zhang et al., 2010). It is unknown whether transferred iTreg can suppress lung inflammation in AAI which is driven by CD4⁺ cells of different antigen reactivity. In order to test this, iTreg and Th2 cells responsive to different unrelated antigens were used and suppression was

determined in the presence of both cognate antigens and Th2 cognate antigen only. WT hosts received 5×10^6 2D2 iTreg prior to AAI induction with 2.5×10^6 Th2 CD4⁺ cells responsive to OVA₃₂₃₋₃₃₉ (OT-II cells) and airway challenges with PBS, OVA or OVA/rMOG (Figure 5.31A). A lower dose of Th2 OT-II cells was used as these were shown to induce significantly more severe inflammation compared to Th2 2D2 cells (Mackenzie, 2011). The capacity of 2D2 iTreg to suppress Th2 2D2 cells was tested and confirmed in the same experiment, however it is not shown in this section (data not shown).

2D2 iTreg did not reduce the total cell numbers in BAL in the AAI model driven by Th2 OT-II cells even when iTreg cognate antigen was present (Figure 5.31B). The percentages of eosinophils and lymphocytes in BAL were unaffected by 2D2 iTreg treatment as well (Figure 5.31C). However, the total number of eosinophils was significantly reduced by 2D2 iTreg and this required the presence of their cognate antigen (Figure 5.31D). The total numbers of macrophages were similar in all groups (Figure 5.31E). Peri-vascular and peri-bronchiolar infiltration scores were unchanged, as were the percentages of goblet cells in small and large airways (Figure 5.32A&B).

Analysis of total cell numbers and percentages of CD4⁺ cells in the lung, medLN and spleen revealed that these were unaffected by the 2D2 iTreg treatment in the presence or absence of their cognate antigen (data not shown). The percentages and total cell numbers of 2D2 iTreg were found to be significantly increased in mice challenged with rMOG/OVA (Figure 5.33A) indicating that the presence of iTreg cognate antigen is critical for their migration to the medLN and the lung and/or survival. The ratio of 2D2 iTreg to Th2 OT-II cells within the lung was very low compared to that observed with Th2 2D2 cells (Figure 5.33B) and although it seemed higher it was not significantly affected by the presence of iTreg cognate antigen. The 2D2 iTreg to Th2 OT-II cell ratios in the medLN and spleen were similar to the one in the lung and significantly higher in the rMOG/OVA-challenged group compared to the OVA only group (Figure 5.33B).

It is important to note that no increase in the percentage of total Foxp3⁺ CD4⁺ cells or Foxp3⁺ Th2 OT-II cells was observed in any of the groups (data not shown).

5.2.2.14. 2D2 iTreg suppress Th2-associated cytokines in a bystander fashion in AAI

Effector functions of Th2 OT-II cells were investigated by overnight re-stimulation of the cells isolated from medLN and the spleen with OVA₃₂₃₋₃₃₉ and MOG₃₅₋₅₅ followed by intracellular cytokine staining. 2D2 iTreg significantly reduced the percentages of IL-13⁺ and IL-5⁺ Th2 OT-II cells within the medLN upon OVA/rMOG challenge (Figure 5.34A). The frequency of IFN- γ ⁺ Th2 OT-II cells was unaffected. Similar pattern of results was observed in the spleen (Figure 5.34B). Interestingly, in the spleen the percentages of IL-13⁺ and IL-5⁺ Th2 OT-II cells were also suppressed by 2D2 iTreg in the absence of their cognate antigen. Insufficient numbers of cells isolated from the lung prevented the analysis of cytokines.

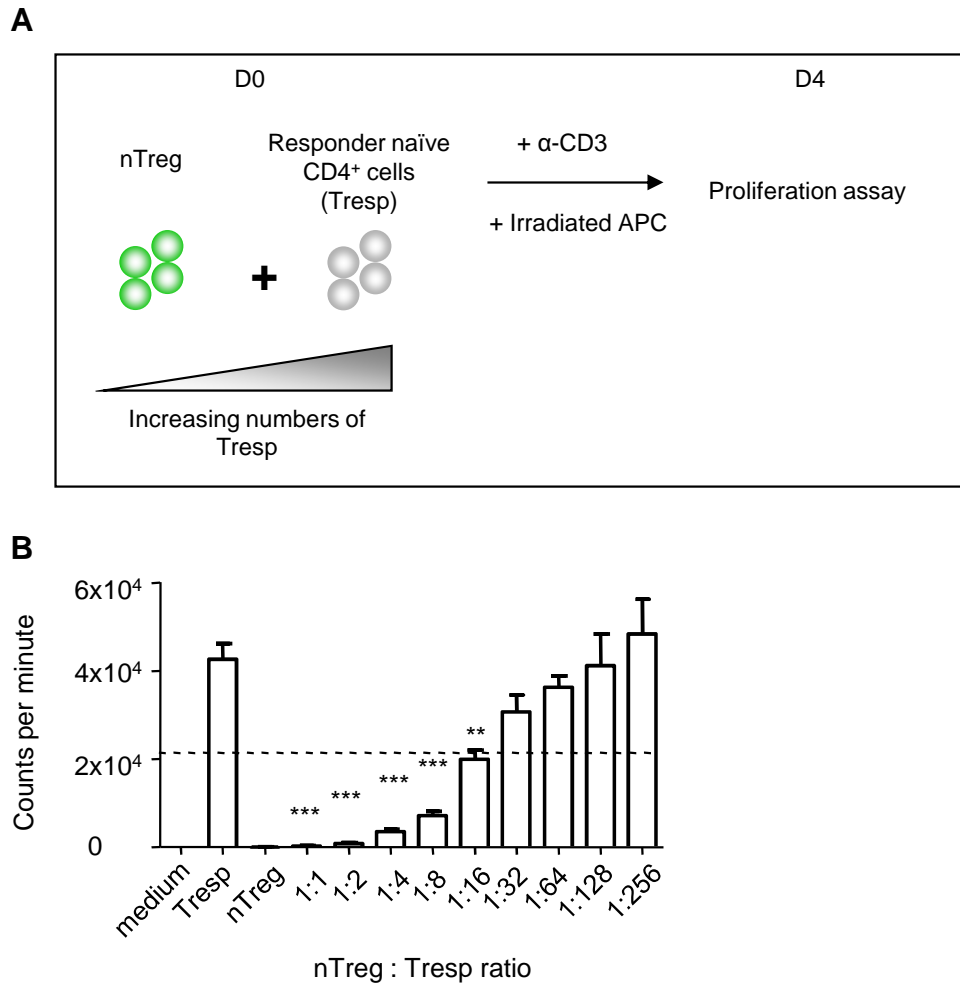


Figure 5.1 Naïve nTreg suppress polyclonal proliferation of naïve CD4⁺ cells *in vitro*.

Freshly isolated nTreg from Foxp3-GFP mouse were mixed with increasing numbers of sorted naïve CD4⁺ cells (Tresp) and stimulated with α -CD3 in the presence of irradiated APC. Proliferation of naïve CD4⁺ cells was measured by ³H thymidine incorporation on day 4. (A) Experimental scheme. (B) Suppression assay showing proliferation of Tresp. Medium only (no α -CD3), Tresp only and nTreg only were used as controls. Dashed line marks 50% of maximum proliferation noted in Tresp only sample. Data are from one experiment, using triplicate wells. ** $p < 0.01$, *** $p < 0.001$ as determined by a one way variance (ANOVA) using Dunnett's multiple comparison test and Tresp sample as a reference.

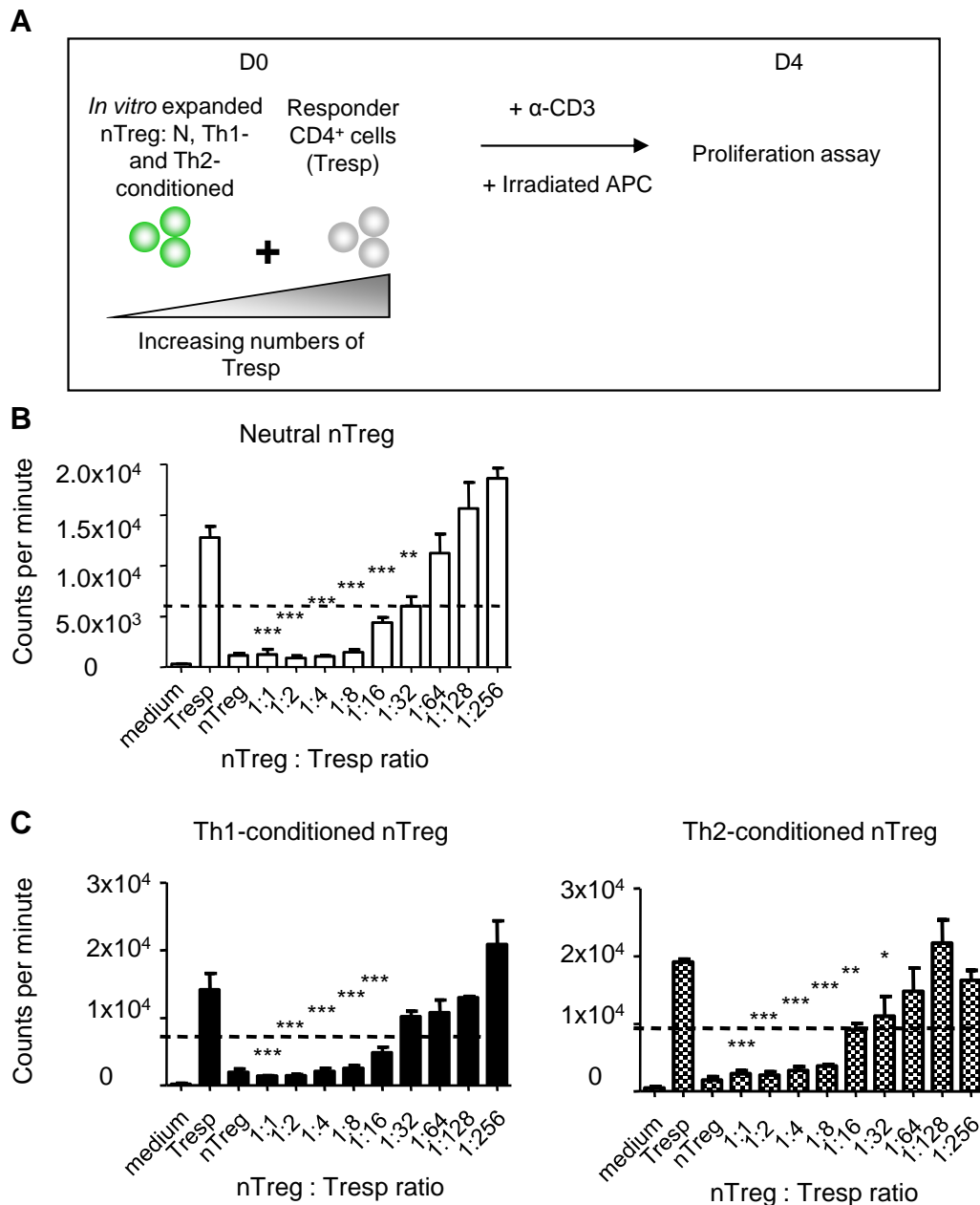


Figure 5.2 nTreg expanded under Th1- and Th2-polarising conditions effectively suppress polyclonal proliferation of naïve CD4⁺ cells *in vitro*.

Sorted GFP⁺ CD4⁺ CD62L^{hi} nTreg from Foxp3-GFP mice were expanded under neutral, Th1 (IL-12, IL-18) or Th2 (IL-4, α -IL-12, α -IFN- γ) conditions for 7 days. They were mixed with increasing numbers of sorted naïve CD4⁺ cells (Tresp) and stimulated with α -CD3 in the presence of irradiated APC. Proliferation of Tresp was measured by ³H thymidine incorporation on day 4. (A) Experimental scheme. (B) Suppression mediated by nTreg expanded under neutral conditions and (C) by nTreg expanded under Th1 and Th2 polarising conditions. Medium only (no α -CD3), Tresp only and nTreg only were used as controls. Dashed lines marks 50% of maximum proliferation noted in Tresp only sample. Data are from one experiment, using triplicate wells. * p <0.05, ** p <0.01, *** p <0.001 as determined by a one way variance (ANOVA) using Dunnett's multiple comparison test and Tresp sample as a reference.

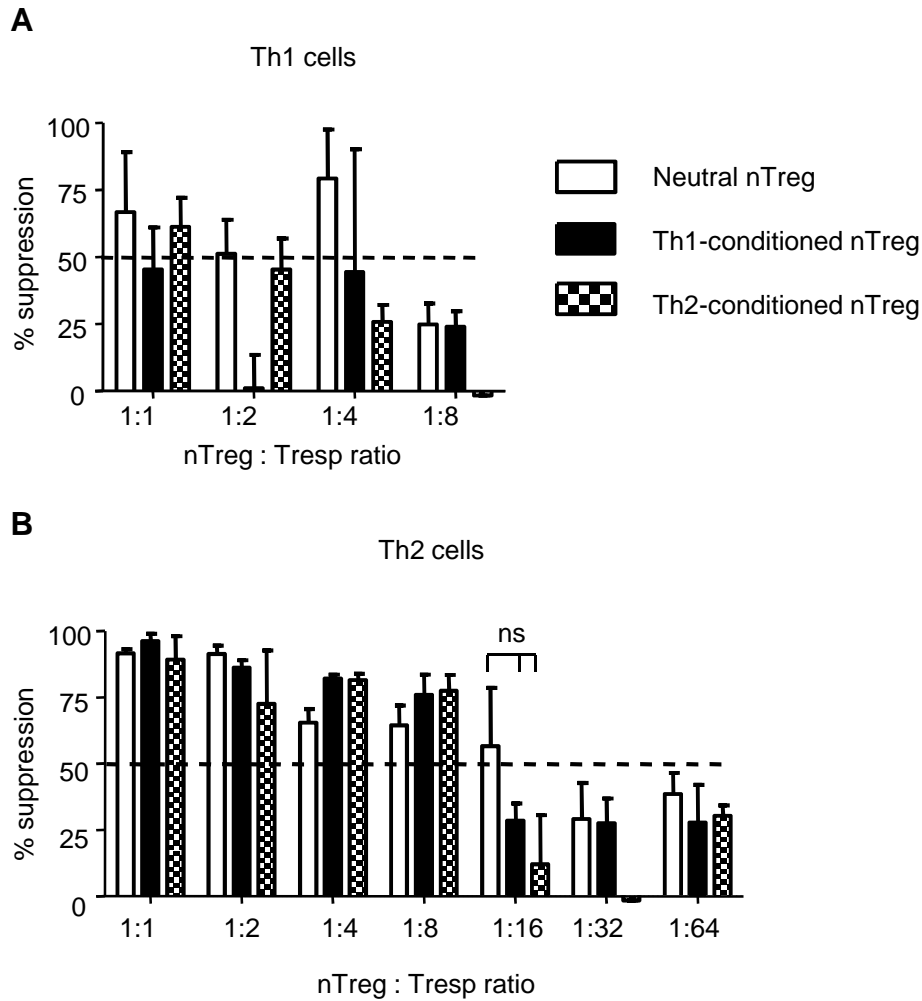


Figure 5.3 nTreg expanded under neutral or polarising conditions do not differ in their ability to suppress pre-activated Th1 and Th2 cells.

Experimental scheme is shown in Figure 5.2. Tresp cells were obtained by polyclonal stimulation of whole splenocytes under Th1 or Th2 polarising conditions followed by 3-day rest in IL-2 and CD4⁺ cell sort. (A) Percentages of suppression of Th1 cell proliferation and (B) Th2 cell proliferation. Dashed line marks 50% of suppression. Data are from one experiment, using duplicate cells with Th1 cells and triplicate wells with Th2 cells. ns=not significant as determined by a one-way variance (ANOVA) using Tukey's multiple comparison test.

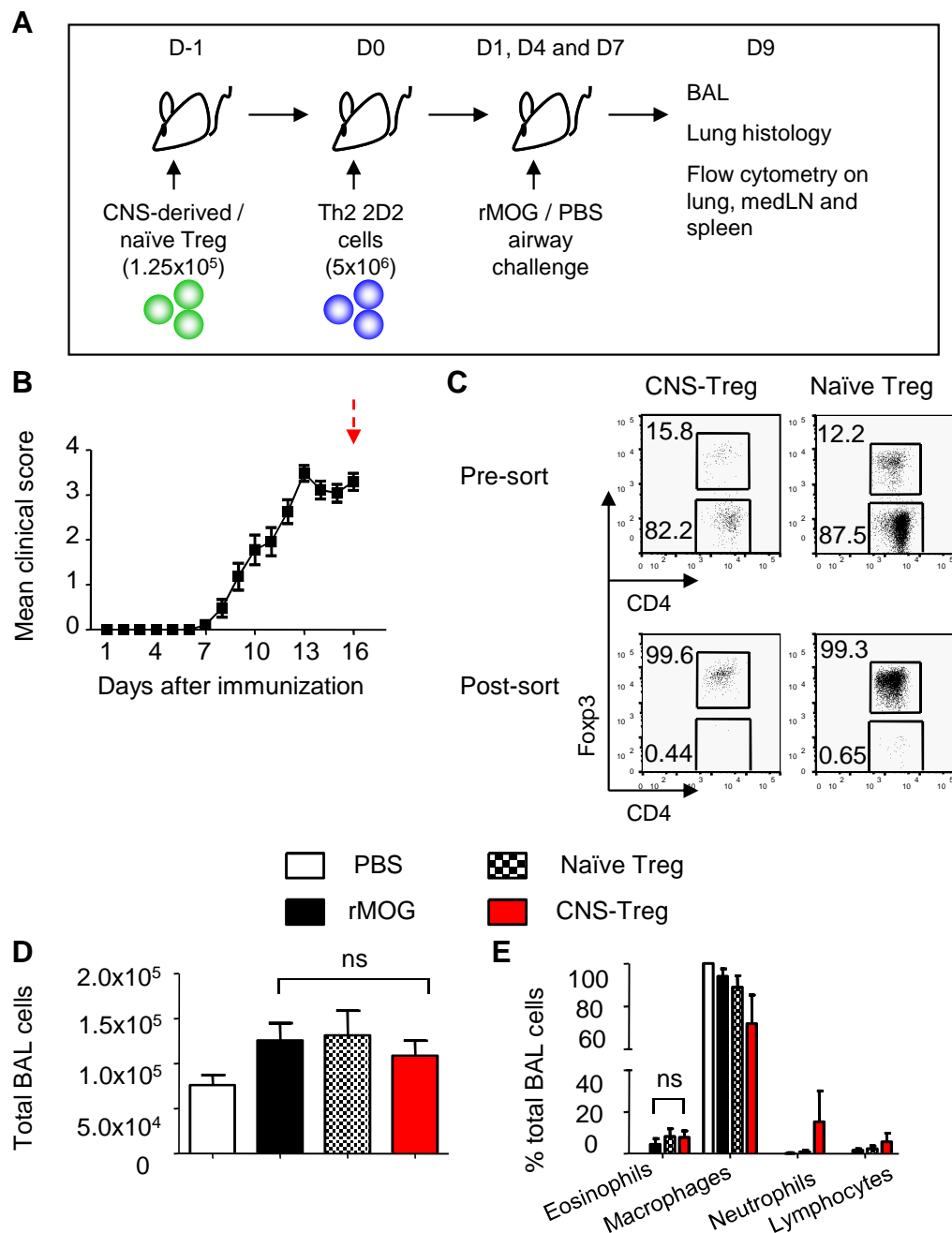


Figure 5.4 CNS-derived Treg do not suppress BAL eosinophilia in rMOG-induced AAI.

EAE was induced in Foxp3-GFP (CD90.2⁺ CD45.2⁺) mice by immunization with MOG₃₅₋₅₅ in CFA. CNS was harvested on day 16, GFP⁺ Treg were sorted and 1.25×10^5 cells were transferred into WT (CD90.2⁺ CD45.1⁺) mice. Treg from naïve Foxp3-GFP spleen were used as a control. The following day, Th2 polarised 2D2 CD4⁺ cells (CD90.1⁺ CD45.2⁺) were transferred followed by three intratracheal challenges with rMOG. (A) Experimental scheme. (B) Mean clinical scores of EAE, $n=27$. Red arrow indicates harvest. (C) Purity of GFP⁺ cells pre- and post-sort using FACS. (D) Total cell numbers in BAL. (E) Percentages of cells in BAL. Data are from one experiment, $n=4$. ns=not significant as determined by a one-way variance (ANOVA) using Tukey's multiple comparison test.

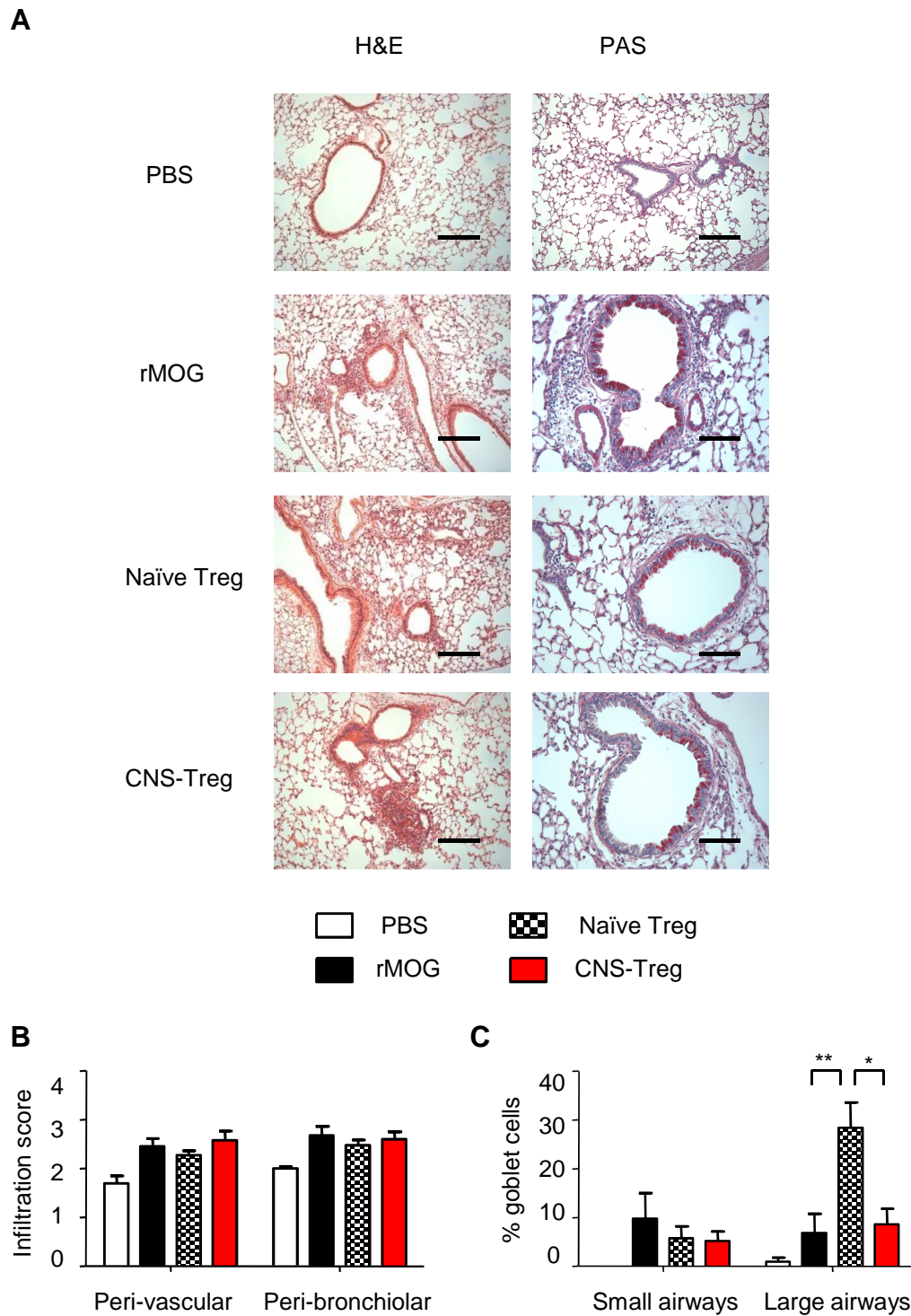


Figure 5.5 CNS-derived Treg do not suppress lung infiltration and goblet cell hyperplasia in rMOG-induced AAI.

Experimental scheme is shown in Figure 5.4. (A) H&E and PAS staining. (B) Infiltration scores and (C) percentages of goblet cells in the lung. Black line represents 100 μm. Data are from one experiment, n=4. *p<0.05, **p<0.01 significant as determined by a one-way variance (ANOVA) using Tukey's multiple comparison test.

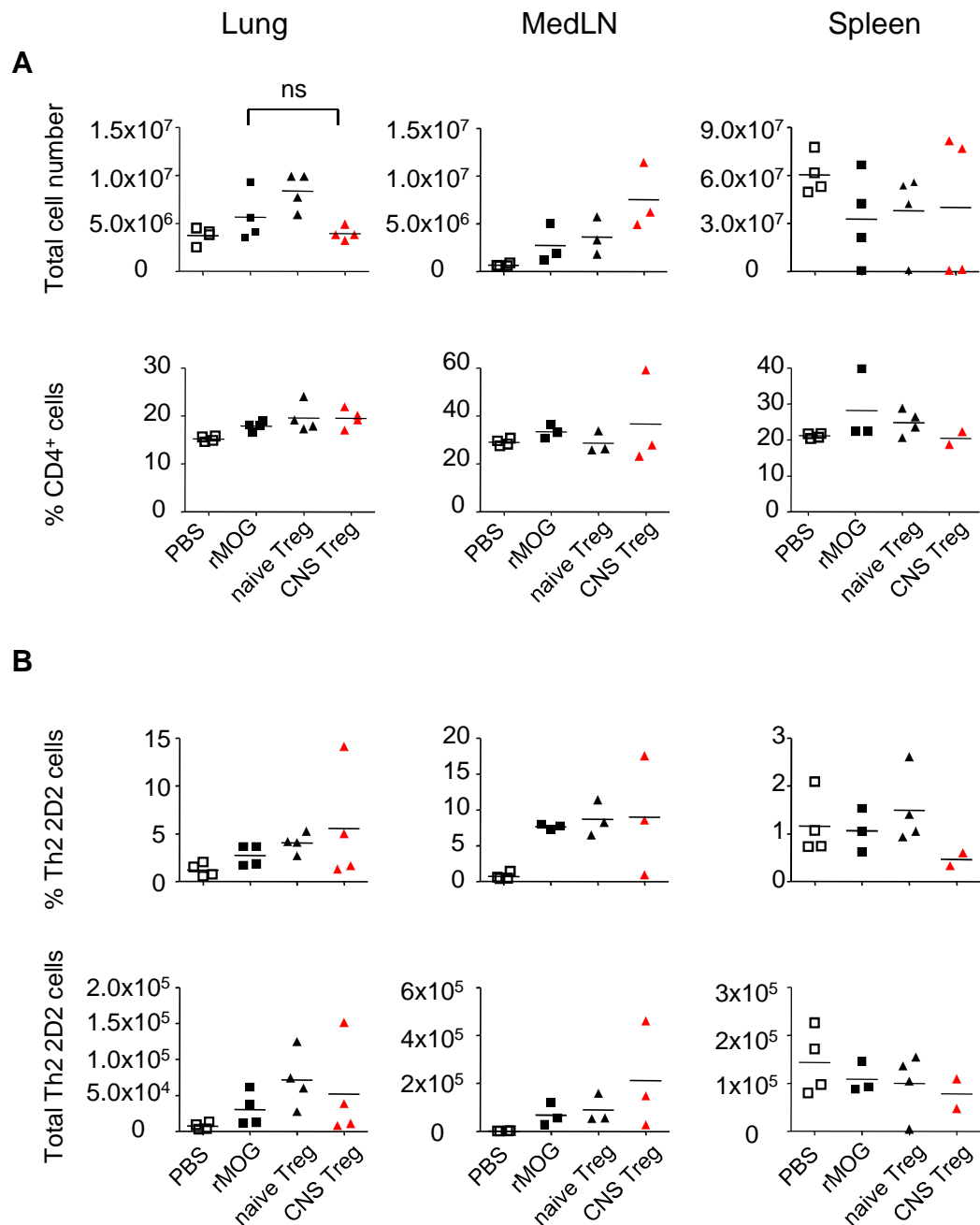


Figure 5.6 Total cell numbers and percentages of Th2 2D2 cells in the lung and medLN are unaffected by CNS-derived Treg.

Experimental scheme is shown in Figure 5.4. (A) Total cell numbers and percentages (%) of CD4⁺ T cells of all cells. (B) Percentages of Th2 2D2 cells of all CD4⁺ cells and total numbers of Th2 2D2 cells. Data are from one experiment, n=4. ns=not significant as determined by a one-way variance (ANOVA) using Tukey's multiple comparison test.

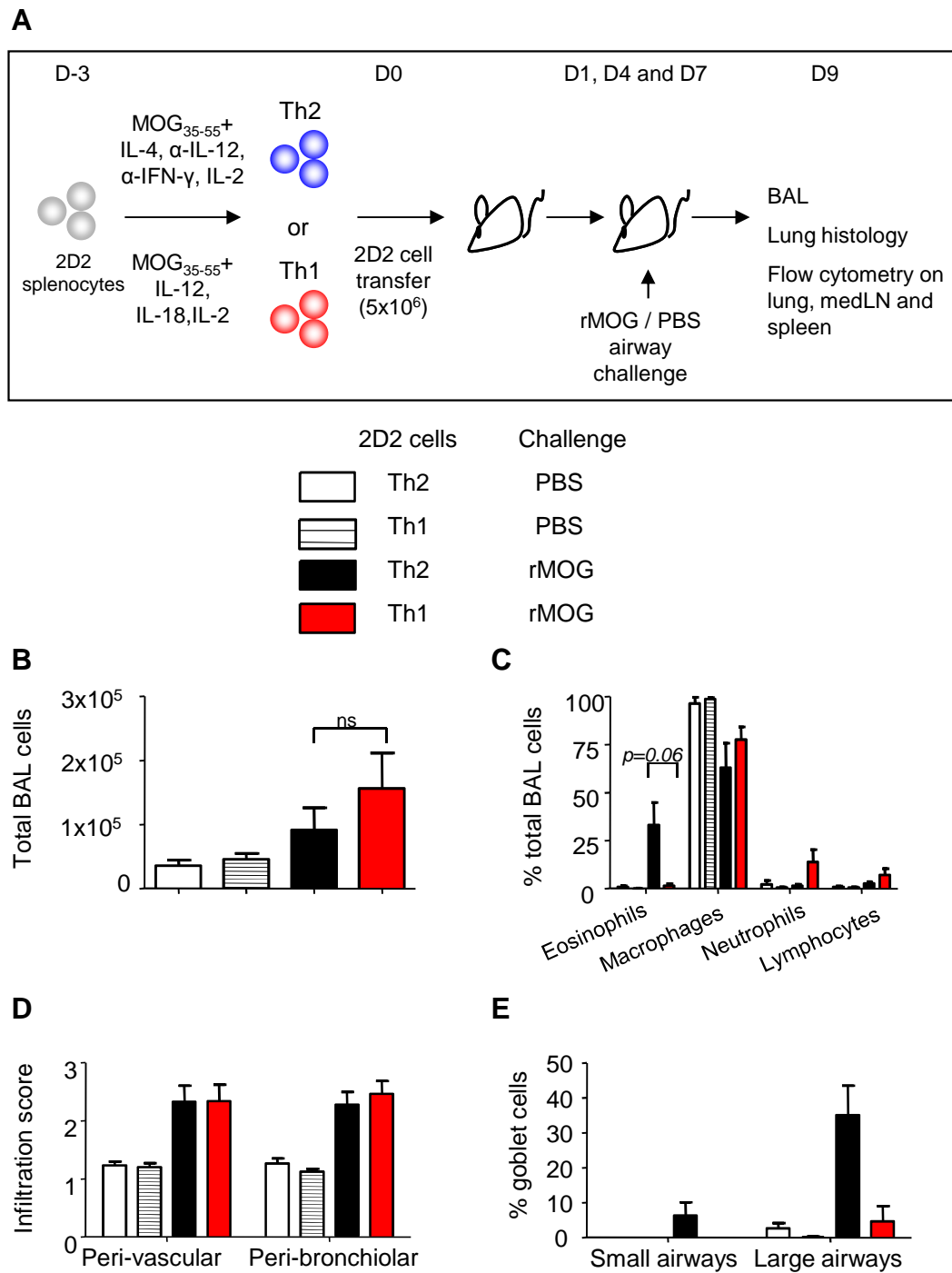


Figure 5.7 Adoptive transfer of Th1 2D2 cells followed by rMOG challenge induces neutrophils in BAL and lung infiltration but not goblet cell hyperplasia.

CD90.1⁺ 2D2 splenocytes were stimulated with MOG₃₅₋₅₅ under Th1 or Th2 polarising conditions, sorted for CD4⁺ cells and 5x10⁶ cells were transferred into WT (CD90.2⁺) mice followed by three intratracheal challenges with rMOG. (A) Experimental scheme. (B) Total cell numbers in BAL. (C) Percentages of cells in BAL. (D) Infiltration scores and (C) percentages of goblet cells in the lung. Data are representative of 2 experiments, n=4-5. Data are shown as mean \pm SEM. ns=not significant as determined by an unpaired t-test.

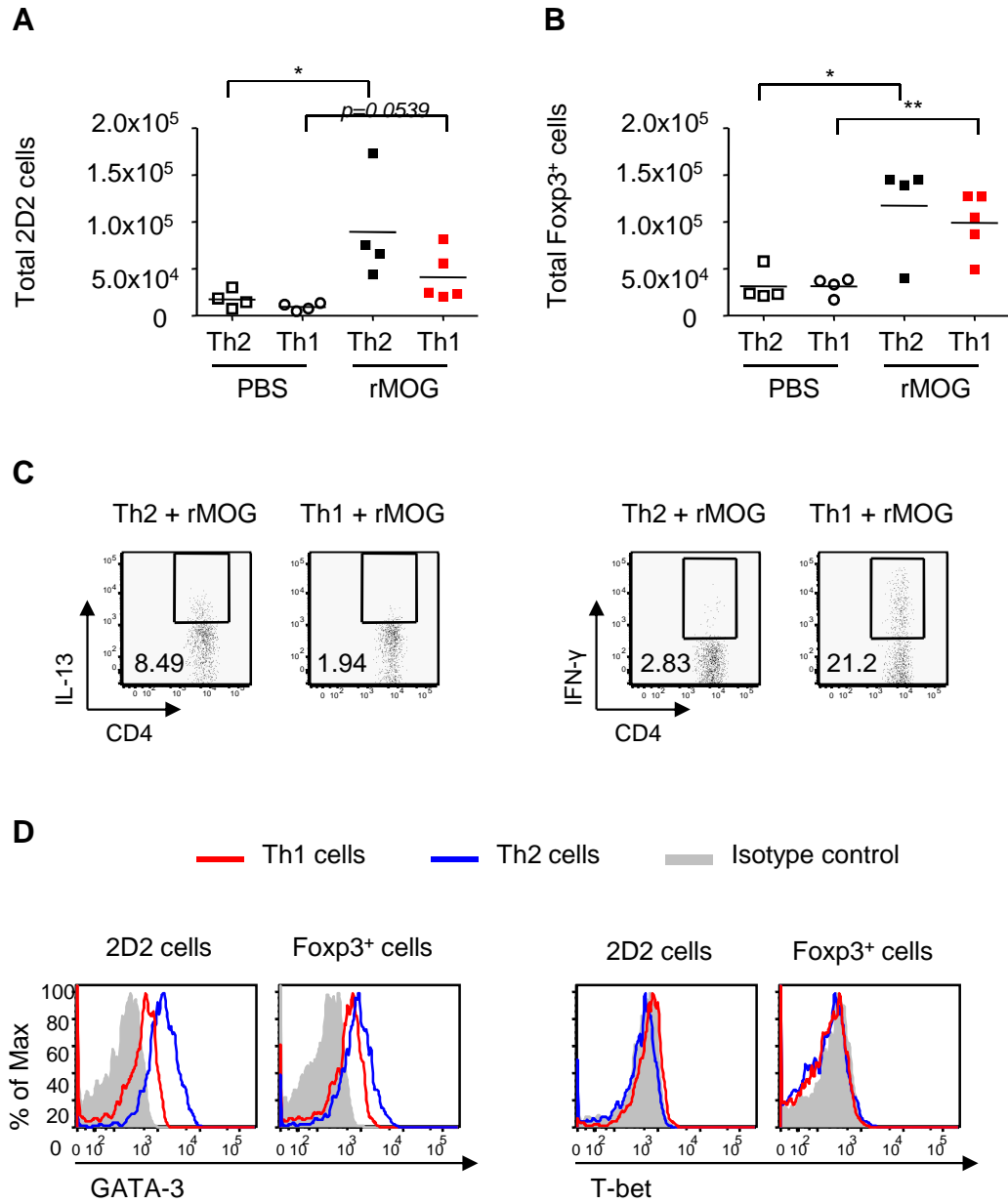


Figure 5.8 Transfer of Th1 2D2 cells induces an increase in the number of Foxp3⁺ cells within the lung however these do not express T-bet.

Experimental scheme is shown in Figure 5.7. (A) Total numbers of transferred 2D2 cells and (B) Foxp3⁺ cells within the lung. (C) Representative flow cytometry plots showing production of IFN- γ and IL-13 by Th1 and Th2 2D2 cells isolated from rMOG-challenged groups and re-stimulated overnight with MOG₃₅₋₅₅. (D) Representative histograms showing expression of GATA-3 and T-bet in transferred 2D2 cells and in Foxp3⁺ cells. Red lines mark Th1 cells, blue lines mark Th2 cells and a shaded area is an isotype control. * $p<0.05$, ** $p<0.01$ as determined by an unpaired t-test.

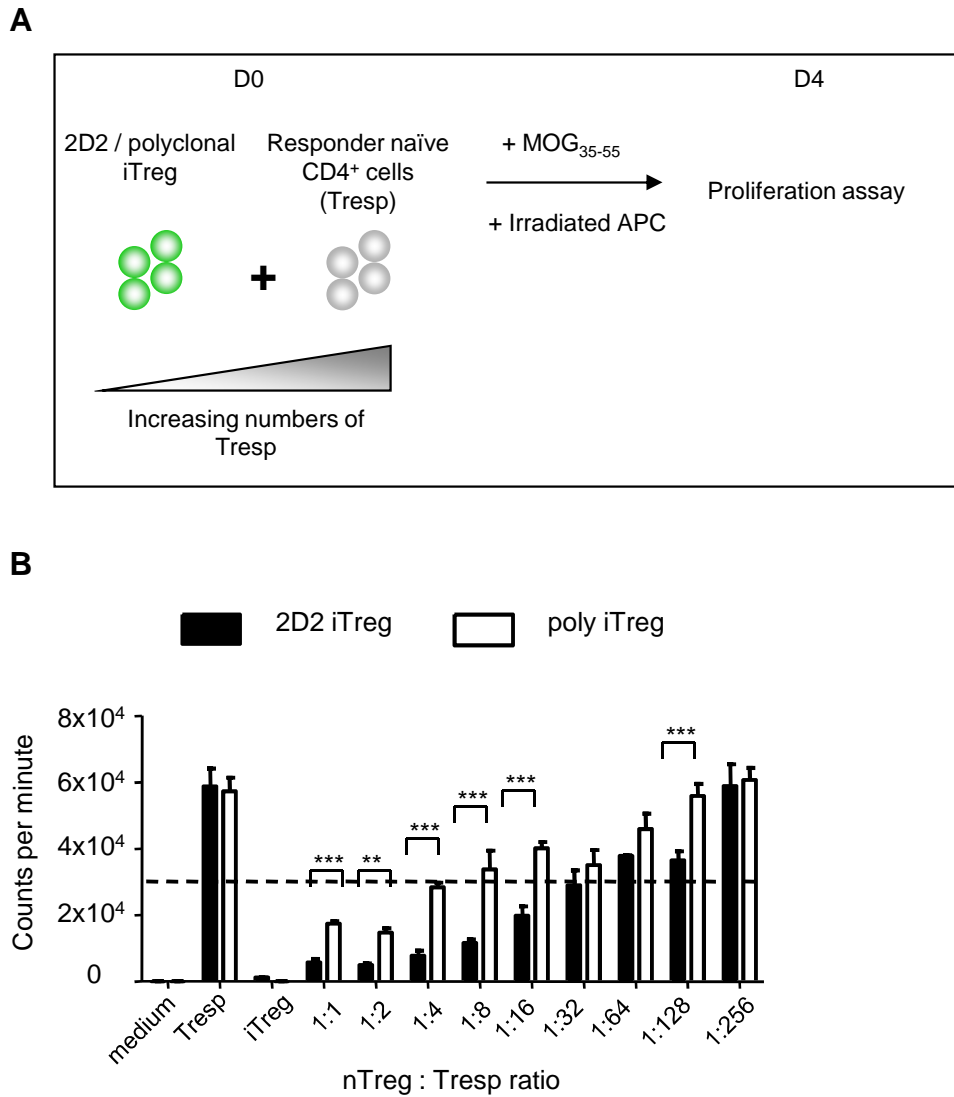


Figure 5.9 2D2 iTreg suppress proliferation of naïve 2D2 CD4⁺ cells *in vitro* more effectively than polyclonal iTreg.

2D2 iTreg and polyclonal iTreg were generated *in vitro* from 2D2xFoxp3.LuciDTR4 and Foxp3.LuciDTR4 mice, respectively, in the presence of TGF- β and IL-2 for 5 days and sorted for GFP⁺ cells using FACS. GFP⁺ cells were mixed with naïve CD4⁺ cells (Tresp) at different ratios and stimulated with MOG₃₅₋₅₅ in the presence of irradiated APC. Proliferation of naïve CD4⁺ cells was measured by ³H thymidine incorporation on day 4. (A) Experimental scheme. (B) Suppression assay showing proliferation of Tresp. Medium only (no MOG₃₅₋₅₅), Tresp only and iTreg only were used as controls. Dashed line marks 50% of maximum proliferation noted in Tresp only sample. Representative of 4 experiments, using triplicate wells. **p<0.01, ***p<0.001 as determined by an unpaired t-test.

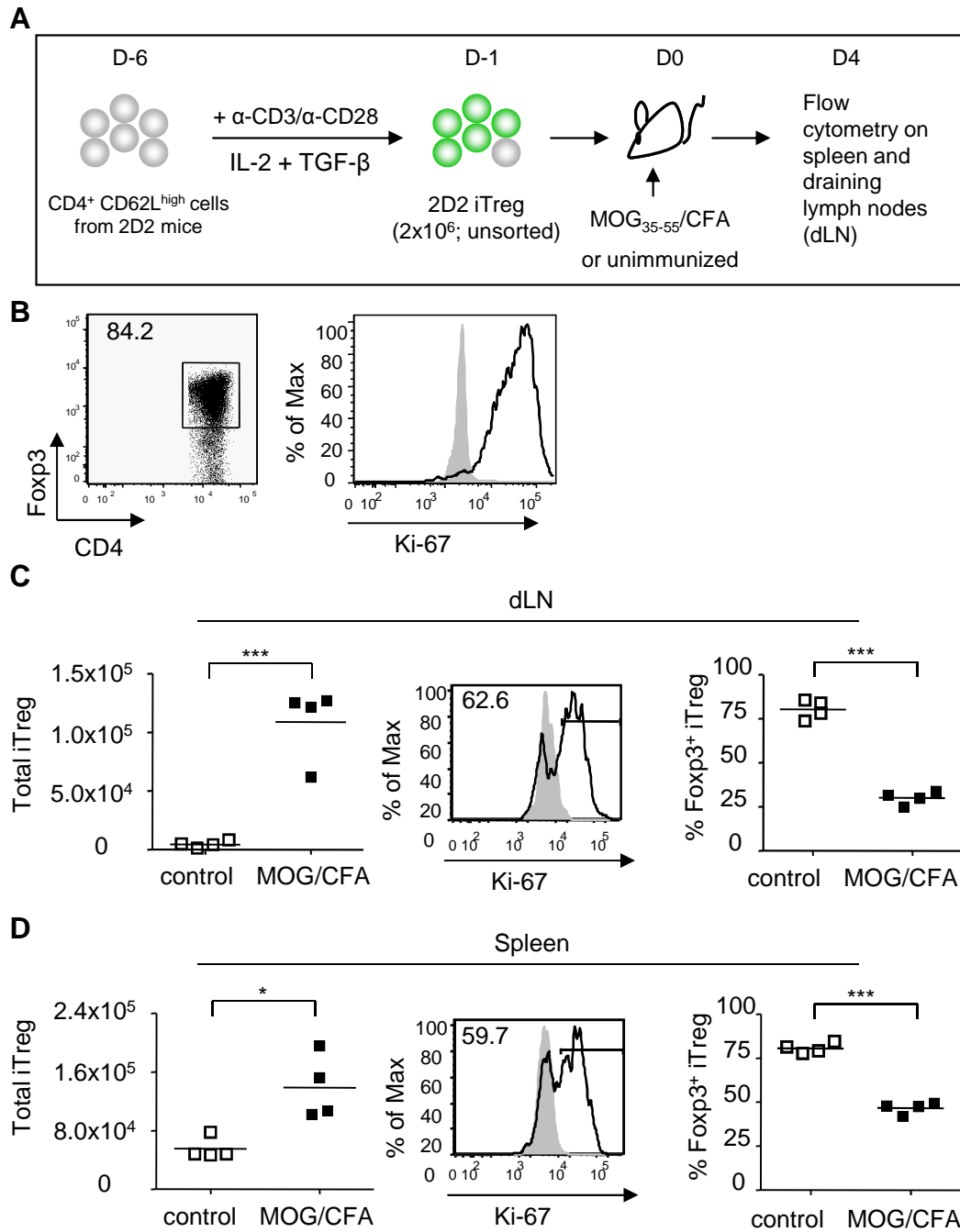


Figure 5.10 2D2 iTreg proliferate *in vivo* in response to antigen and partially lose Fcγ3 expression.

2D2 iTreg (CD90.1⁺CD90.2⁺) were induced from naïve 2D2 CD4⁺ CD62L^{high} cells in the presence of TGF- β and IL-2 for 5 days. On day -1, 2x10⁶ 2D2 iTreg were injected into WT (CD90.2⁺) host followed by immunization with MOG₃₅₋₅₅/CFA. Unimmunized host was used as a control. On day 4, draining LN (dLN) and spleen were harvested. (A) Experimental scheme. (B) Profile of 2D2 iTreg prior to transfer. Naïve CD4⁺ cells are shown in filled histogram. (C) Total numbers of 2D2 iTreg, their expression profile of Ki-67 (gated on all iTreg) and percentages (%) of Fcγ3⁺ iTreg of total iTreg in dLN and D) spleen. Unimmunized control is shown in filled histogram. Data are from one experiment, n=4. *p<0.05, p<0.001 as determined by an unpaired t-test.

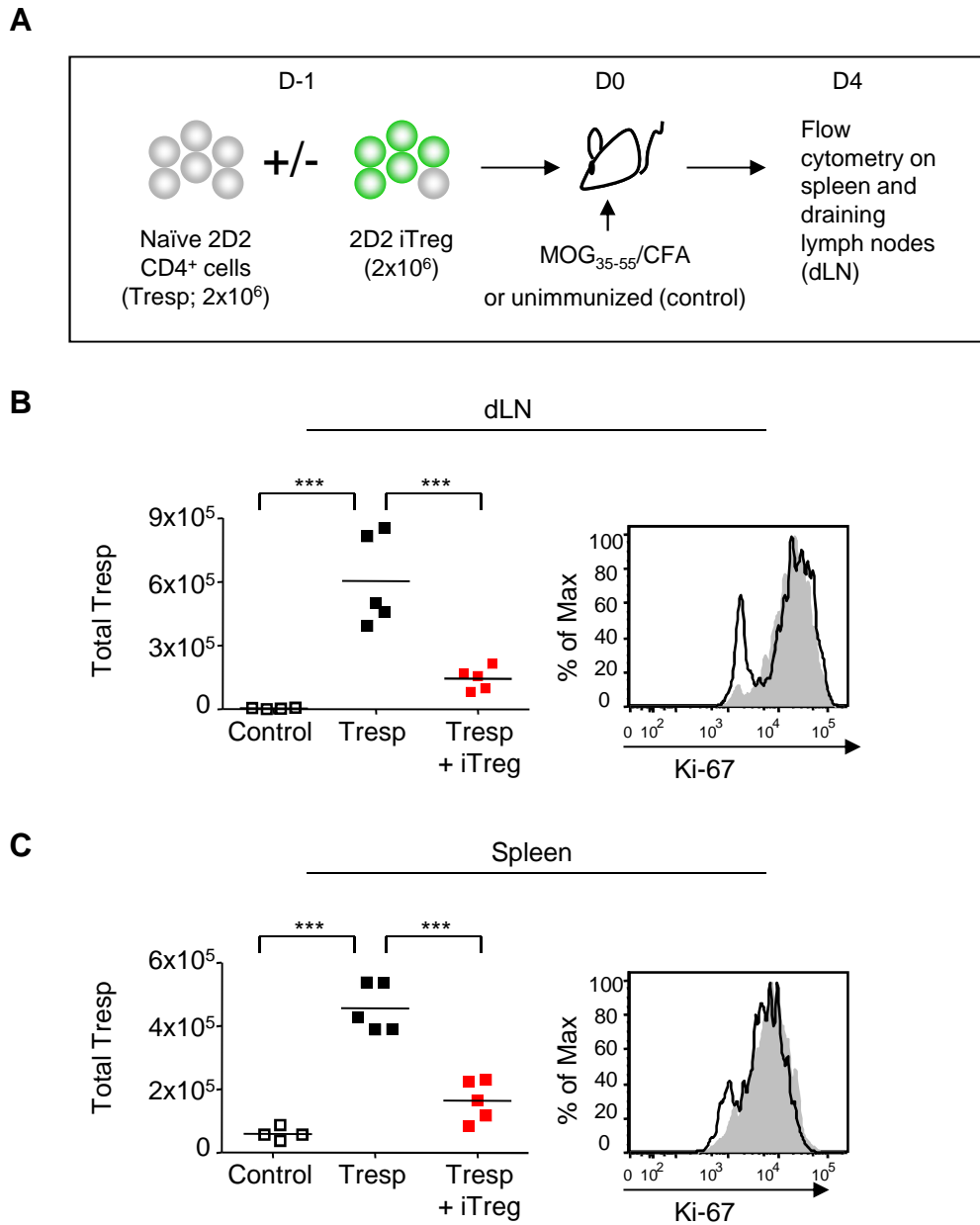


Figure 5.11 2D2 iTreg limit survival but not proliferation of naïve 2D2 CD4⁺ cells *in vivo* upon immunization.

Naïve 2D2 (CD90.1⁺) CD4⁺ cells (Tresp; 2x10⁶) were transferred with or without 2D2 iTreg (CD90.1⁺ CD90.2⁺) into WT (CD90.2⁺) host followed by MOG₃₅₋₅₅/CFA immunization. Unimmunized host that received both naïve 2D2 CD4⁺ cells and 2D2 iTreg was used as a control. On day 4, dLN and spleen were harvested. (A) Experimental scheme. (B) Total cell numbers and Ki-67 staining of Tresp in dLN and (C) in spleen. Black line marks Tresp + iTreg, filled histogram denotes Tresp only. Data represent 2 experiments. ***p<0.001 as determined by a one-way variance (ANOVA) using Tukey's multiple comparison test.

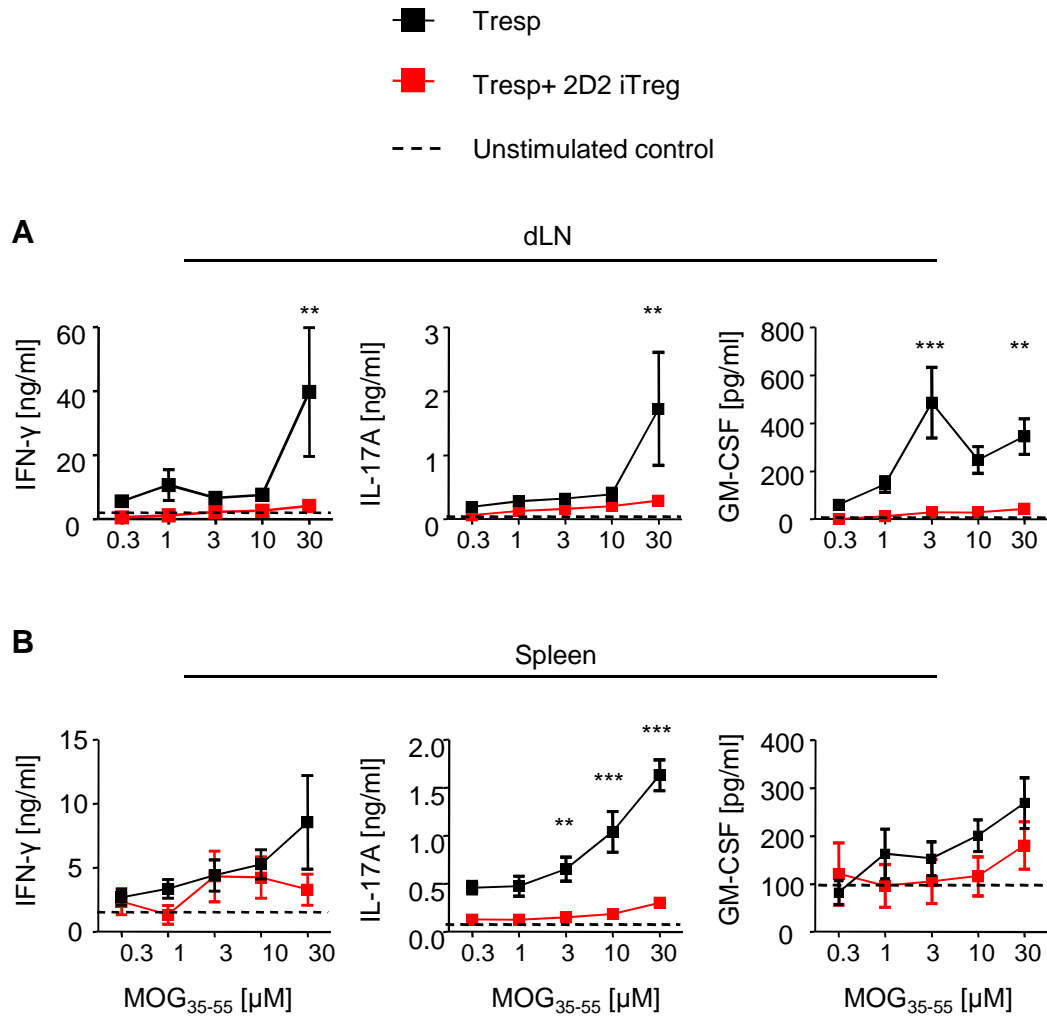


Figure 5.12 2D2 iTreg reduce levels of effector cytokines produced in response to recall stimulation with antigen.

Experimental scheme is shown in Figure 5.11. Cells isolated from dLN and spleen were cultured *in vitro* with increasing concentrations of MOG₃₅₋₅₅; IFN-γ, IL-17A and GM-CSF production was measured from the cell supernatants using ELISA on day 3 of re-stimulation. Dotted lines represent un-stimulated controls. Data are from one experiment. **p<0.01, ***p<0.001 as determined by a two way variance (ANOVA) using Bonferroni multiple comparison test.

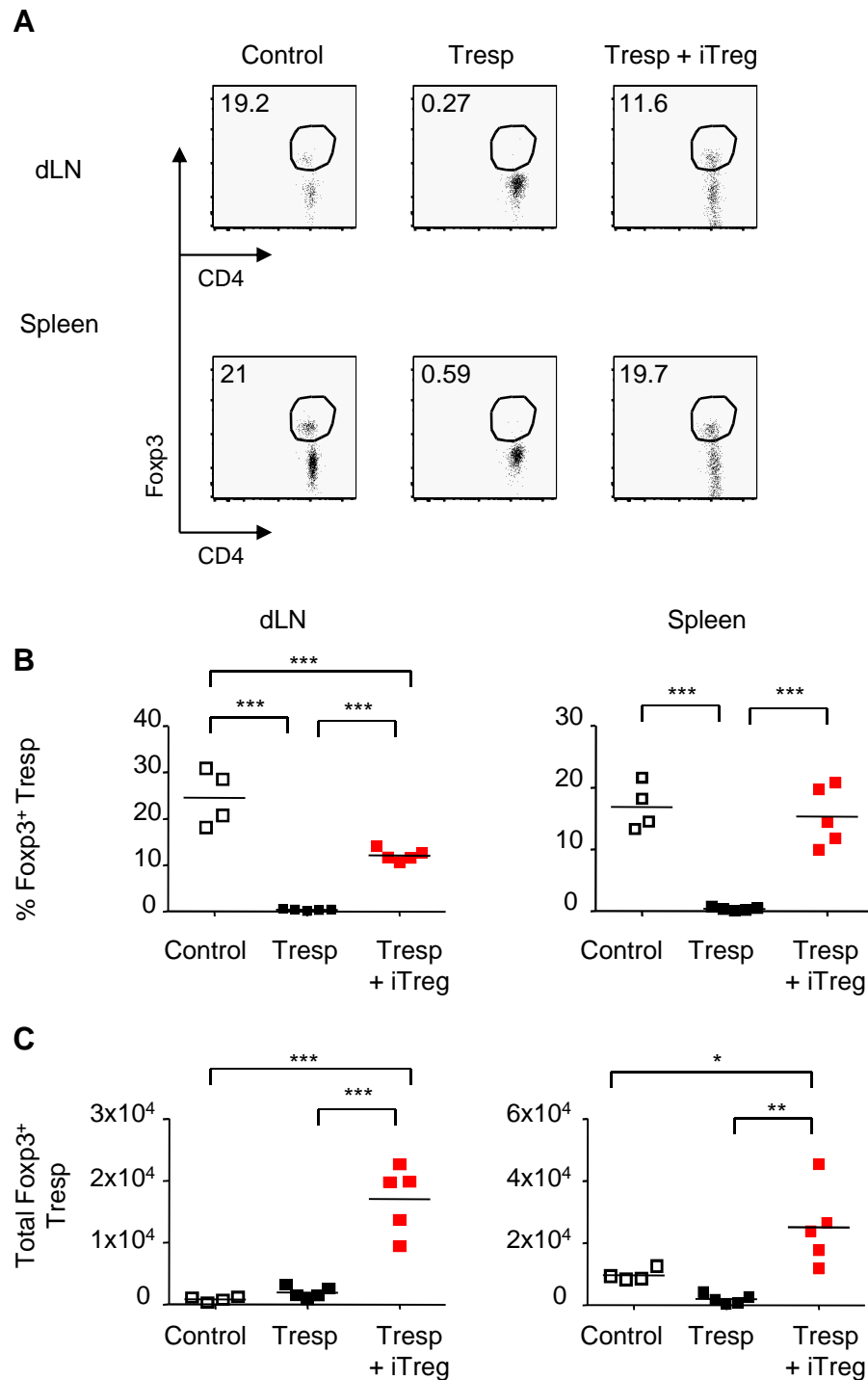


Figure 5.13 2D2 iTreg increase Foxp3 expression within transferred naïve 2D2 CD4⁺ cell population upon immunization.

Experimental scheme is shown in Figure 5.11. (A) Representative histograms showing Foxp3 expression in Tresp. Numbers indicate percentages of gated cells within Tresp population. Control denotes unimmunized host that received both naïve 2D2 CD4⁺ cells and 2D2 iTreg. (B) Percentages (from all Tresp) and (C) total numbers of Foxp3⁺ Tresp. Data are from one experiment. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as determined by a one-way variance (ANOVA) using Tukey's multiple comparison test.

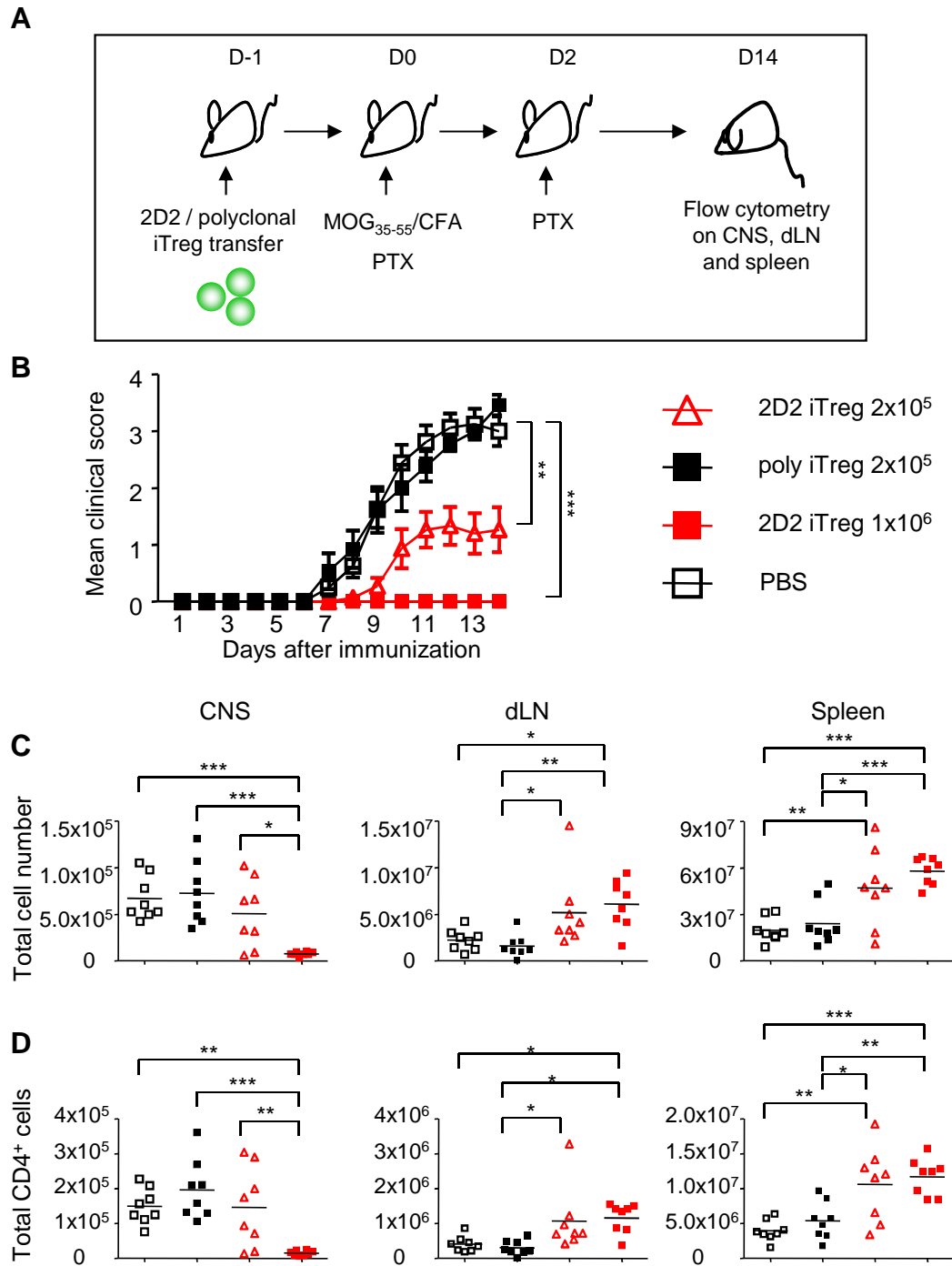


Figure 5.14 2D2 iTreg but not polyclonal iTreg suppress induction of EAE.

FACS sorted GFP⁺ 2D2 or polyclonal iTreg (CD90.1⁺) were transferred into WT (CD90.2⁺) host followed by MOG₃₅₋₅₅/CFA immunization for EAE induction and monitored for clinical signs of disease. Mice were harvested on day 14. (A) Experimental scheme. (B) Clinical course of EAE. Results are from 2 pooled experiments n=8-16. (C) Total cell numbers. (D) Total numbers of CD4⁺ cells. Results are representative of 2 experiments, n=8. ***p<0.001, **p<0.01 as determined by (B) a Fisher's exact test, comparing proportions of mice with a score of ≤2 and a score of ≥3 and (C&D) a one-way variance (ANOVA) using Tukey's multiple comparison test.

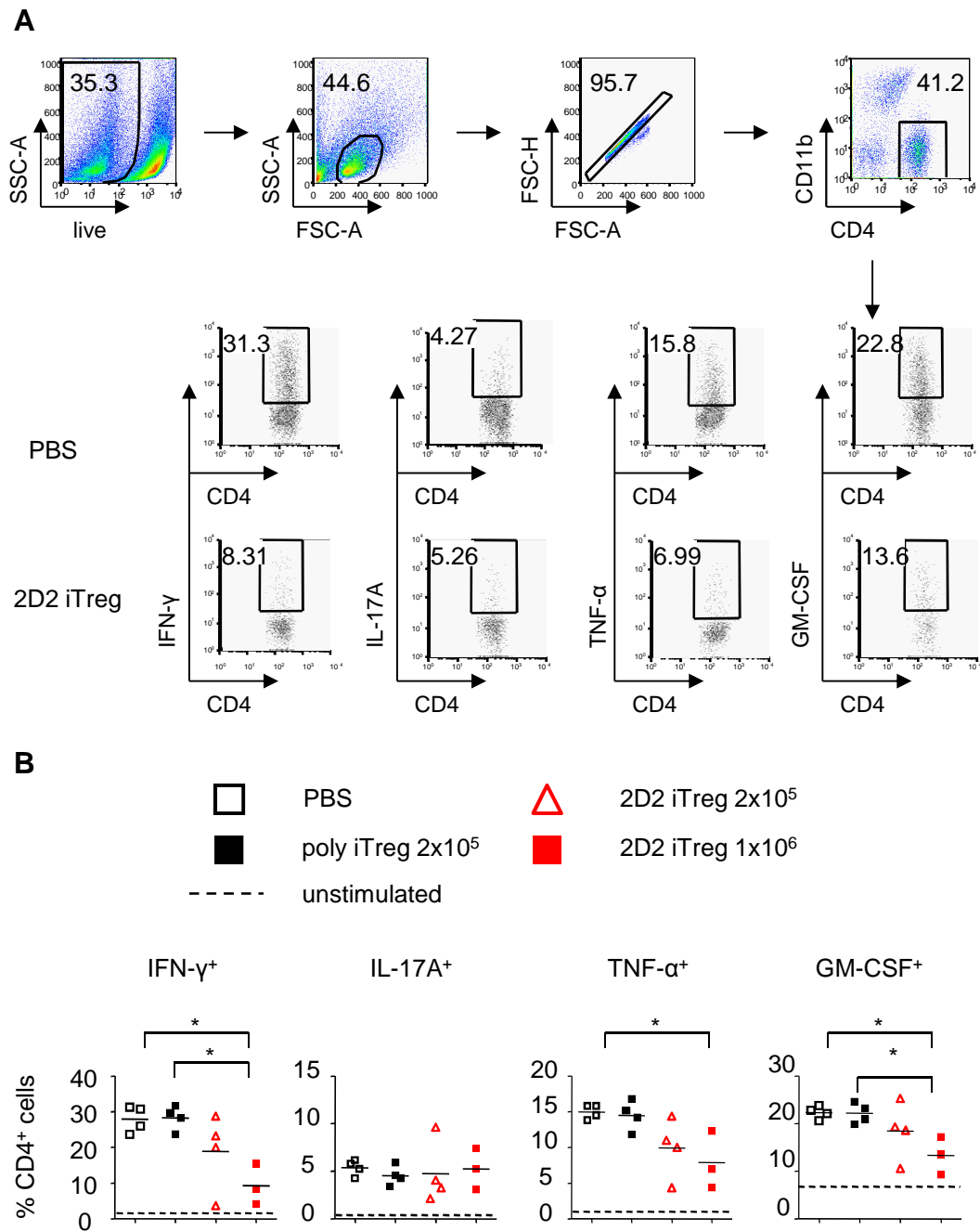


Figure 5.15 2D2 iTreg transferred prior to immunization suppress production of pro-inflammatory cytokines within the CNS.

Experimental scheme is shown in Figure 5.14. Cells isolated from the CNS were cultured overnight in the presence or absence of MOG₃₅₋₅₅ followed by ICS cytokine staining. (A) Gating strategy and representative histograms showing cytokine staining in the CNS of mice that were treated either with PBS or 1×10^6 2D2 iTreg. Numbers indicate percentages of gated cells within CD4⁺ cell population. B) Percentages of CD4⁺ cells expressing IFN-γ, IL-17A, TNF-α or GM-CSF of total CD4⁺ cells in the CNS. Data are representative of 2 experiments, n=8 with each data point representing 2 pooled mice. *p<0.05 as determined by a one-way variance (ANOVA) using Tukey's multiple comparison test.

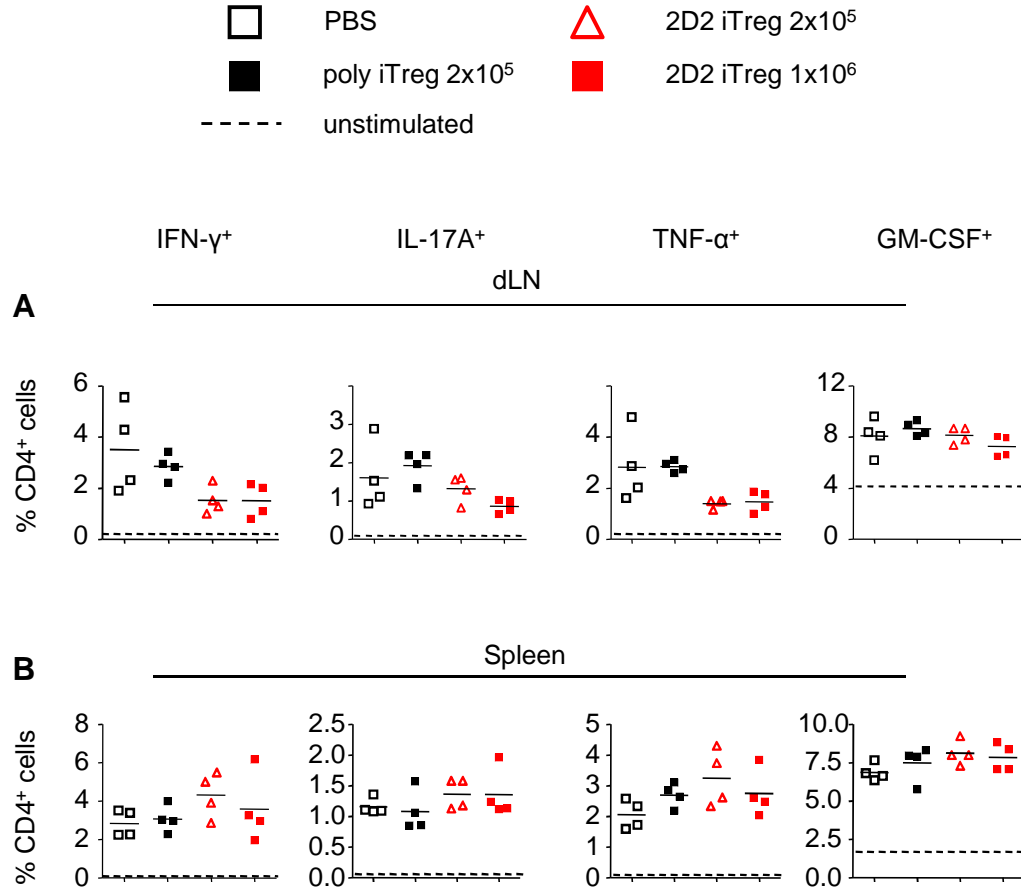


Figure 5.16 2D2 iTreg transferred prior to immunization reduce cytokine production within the dLN but not in the spleen.

Experimental scheme is shown in Figure 5.14. Cells isolated from the dLN and spleen were cultured overnight in the presence or absence of MOG₃₅₋₅₅ followed by ICS cytokine staining. A) Percentages of CD4 $^+$ cells expressing IFN- γ , IL-17A, TNF- α or GM-CSF of total CD4 $^+$ cells in dLN and B) in spleen. Dashed lines mark unstimulated control. Data are representative of 2 experiments, n=8 with each data point representing 2 pooled mice.

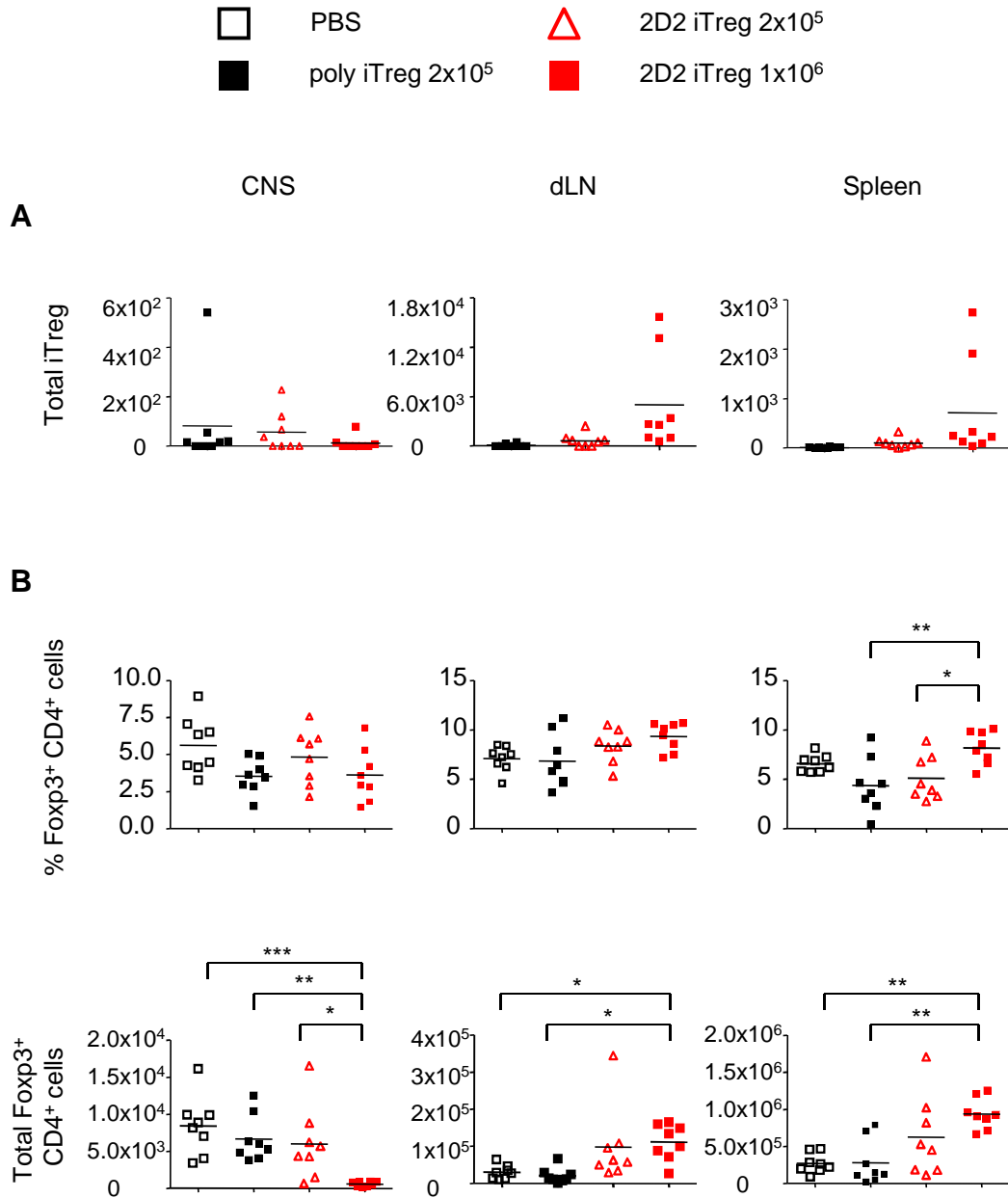


Figure 5.17 2D2 iTreg accumulated within dLN and this corresponded with increased total cell numbers of CD4⁺ Foxp3⁺ cells.

Experimental scheme is shown in Figure 5.14. (A) Total numbers of iTreg. (B) Percentages and absolute numbers of total Foxp3⁺ CD4⁺ cells. Data are representative of 2 experiments, n=8. *p<0.5, **p<0.01, ***p<0.001 as determined by a one-way variance (ANOVA) using Tukey's multiple comparison test.

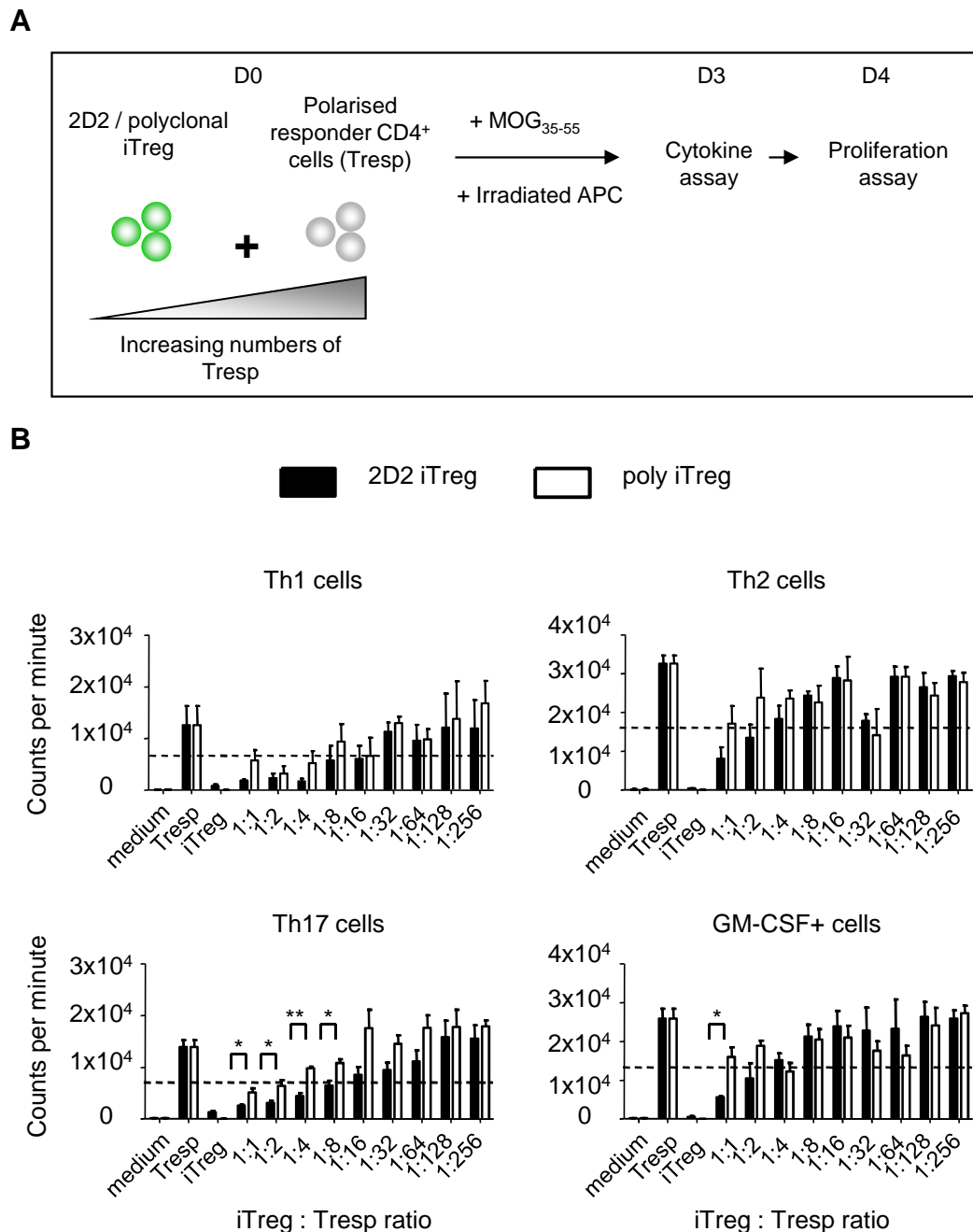


Figure 5.18 2D2 iTreg suppress proliferation of pre-activated polarised CD4⁺ cells *in vitro* more effectively than polyclonal iTreg.

2D2 iTreg and polyclonal iTreg were induced in the presence of TGF- β and IL-2 for 5 days and sorted for GFP⁺ cells using FACS. These were mixed with sorted pre-activated CD4⁺ cells (Tresp) at different ratios and stimulated with MOG₃₅₋₅₅ in the presence of irradiated APC. Proliferation of Tresp was measured by ³H thymidine incorporation on day 4. (A) Experimental scheme. (B) Suppression assay showing proliferation of Tresp. Medium only (no MOG₃₅₋₅₅), Tresp only and iTreg only were used as controls. Dashed lines mark 50% of maximum proliferation noted in Tresp only sample. Data are representative of 4 experiments, using triplicate wells. * $p < 0.05$, ** $p < 0.01$ as determined by an unpaired t-test.

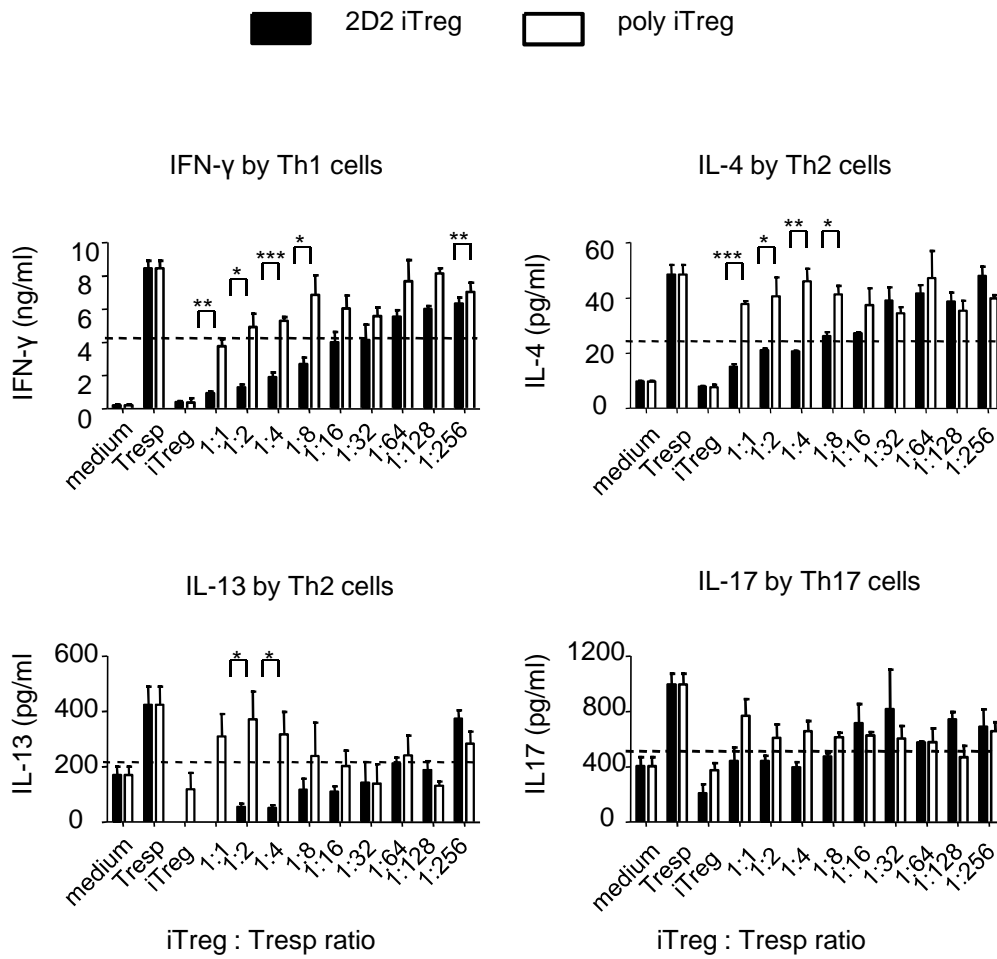
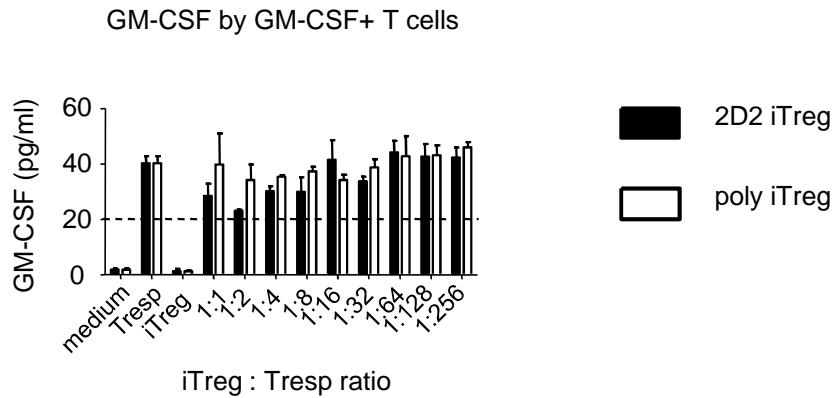


Figure 5.19 2D2 iTreg suppress cytokine production by pre-activated polarised CD4⁺ cells *in vitro* more effectively than polyclonal iTreg.

Experimental scheme is shown in Figure 5.18. Cytokine production by Tresp was measured by ELISA on day 3. Dashed lines mark 50% of maximum cytokine concentration noted in Tresp only sample. Data are representative of 4 experiments, using triplicate wells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as determined by an unpaired t-test.

A



B

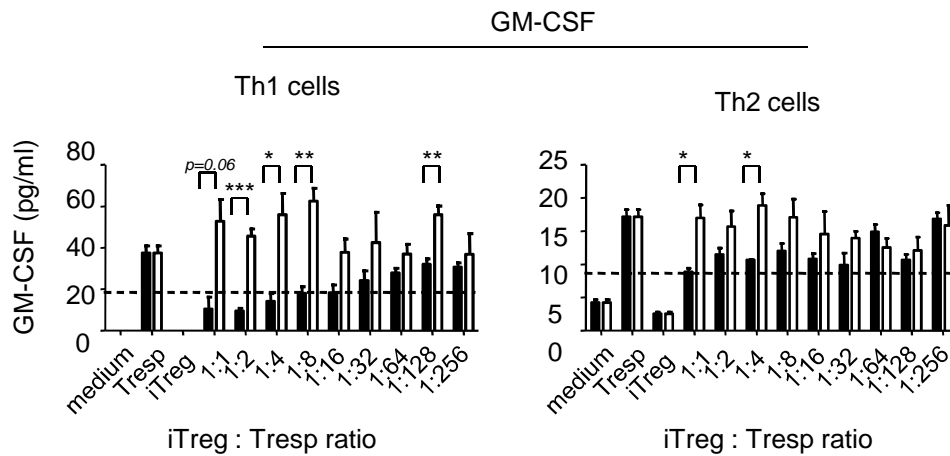


Figure 5.20 2D2 iTreg suppress GM-CSF production by Th1- and Th2-polarised cells but less effectively by GM-CSF-polarised cells.

Experimental scheme is shown in Figure 5.18. Cytokine production by Tresp was measured by ELISA on day 3. (A) GM-CSF production by GM-CSF-polarised cells and by (B) Th1- and Th2-polarised cells. Dashed lines mark 50% of maximum cytokine concentration noted in Tresp only sample. Data are representative of 2 experiments, using triplicate wells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as determined by an unpaired t-test.

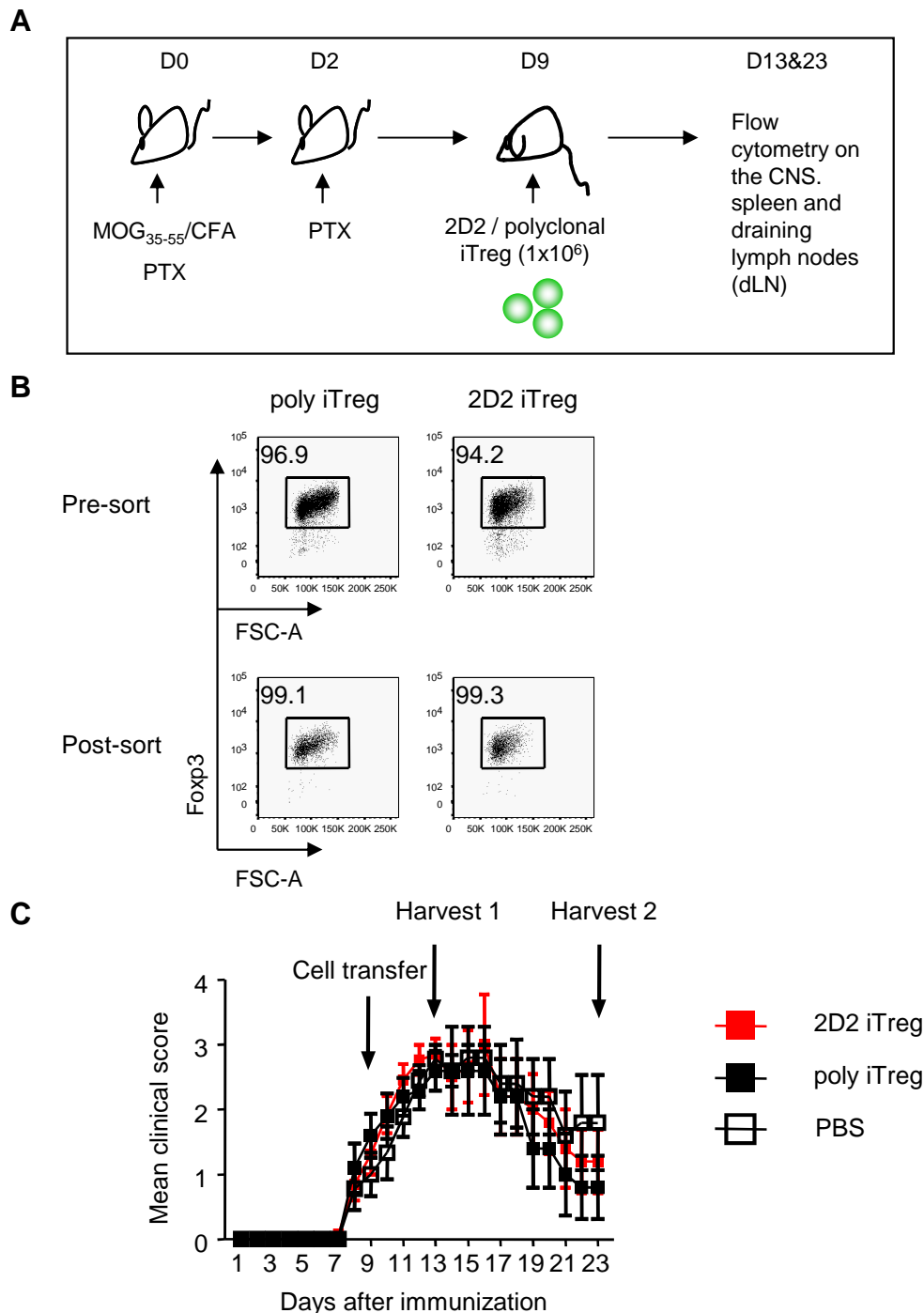


Figure 5.21 2D2 iTreg do not protect from EAE when transferred after the onset of the disease.

WT (CD90.2⁺) hosts were immunized with MOG₃₅₋₅₅/CFA for the induction of EAE and monitored for clinical signs of disease. Upon approaching grade 1-2, mice received intravenous injection of 1×10^6 CD90.1⁺ polyclonal iTreg (poly iTreg) or 2D2 iTreg which were FACS sorted for GFP⁺ cells prior to transfer. PBS was used as a control. Tissue was harvested 4 days and 14 days after iTreg transfer. (A) Experimental scheme. (B) Flow cytometry plots showing percentages of Fop3⁺ (GFP⁺) cells from all cells pre- and post-sort using FACS prior to transfer. (C) Clinical course of EAE. Data are from one experiment, $n=10$.

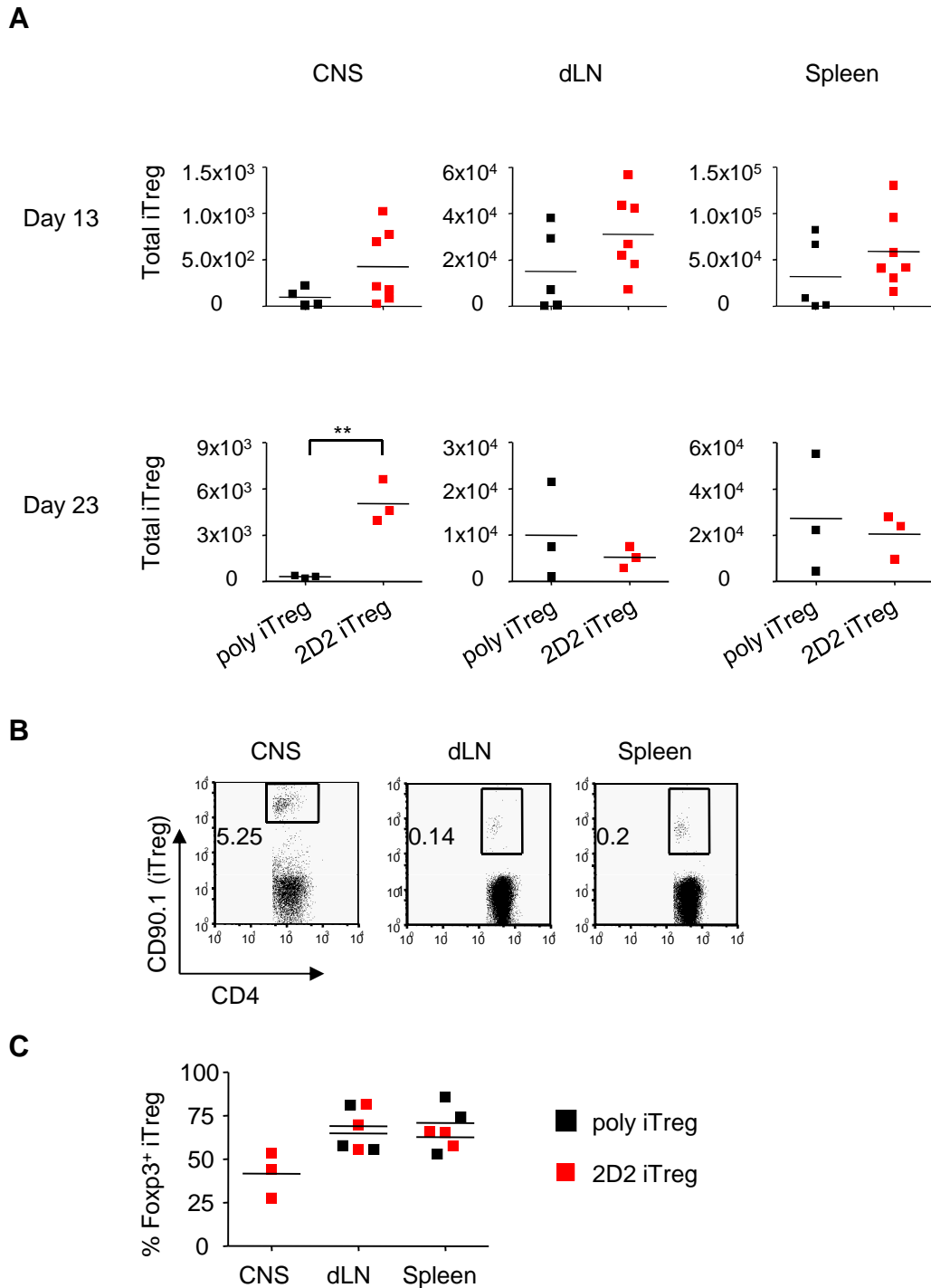


Figure 5.22 2D2 iTreg accumulate in CNS during EAE but are unable to suppress on-going inflammation.

Experimental scheme is shown in Figure 5.21. (A) Total numbers of iTreg on day 13 and day 23. (B) Representative flow cytometry plots showing percentages of iTreg within CD4⁺ cell population on day 23. (C) Foxp3 expression by poly iTreg and 2D2 iTreg on day 23. Foxp3 expression by poly iTreg in the CNS is not shown due to insufficient cell numbers. Data are from one experiment, n=10. **p<0.01 as determined by an unpaired t-test.

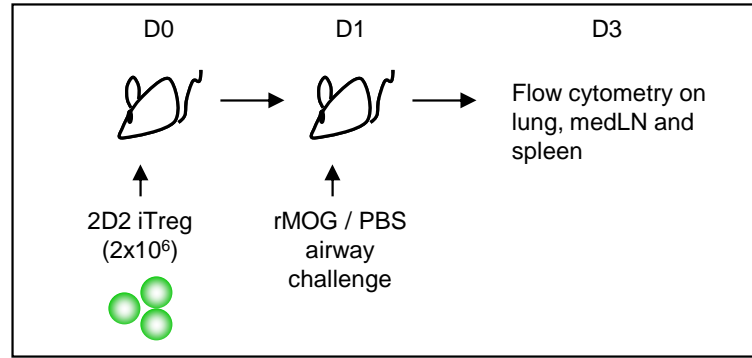
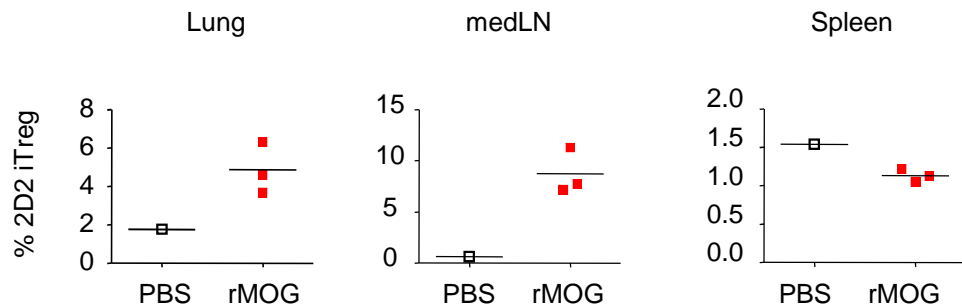
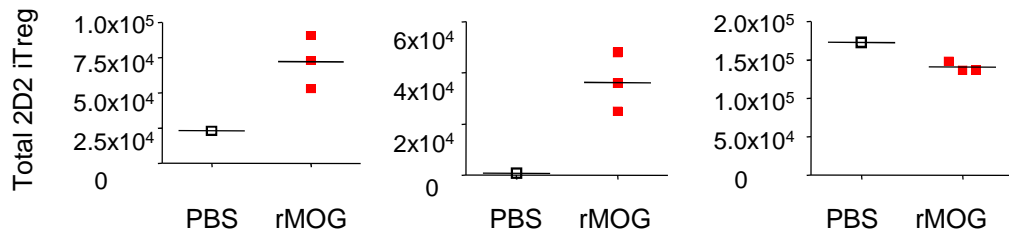
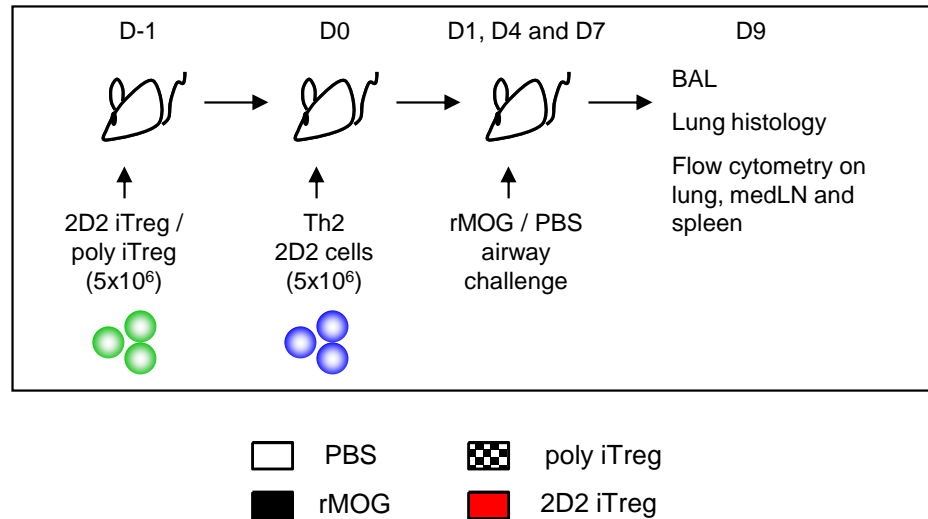
A**B****C**

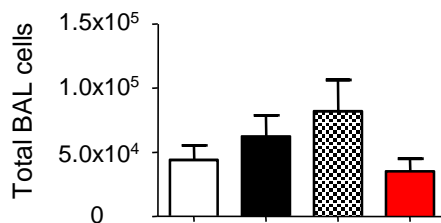
Figure 5.23 2D2 iTreg migrate to the medLN and the lung upon airway challenge with rMOG.

2D2 iTreg ($CD90.1^+$) were transferred into WT ($CD90.2^+$) host followed by a single airway challenge with rMOG. Tissue was harvested 2 days after the challenge. (A) Experimental scheme. (B) Percentages and (C) total numbers of 2D2 iTreg in the lung, medLN and spleen. Data are from one experiment, $n=1-3$.

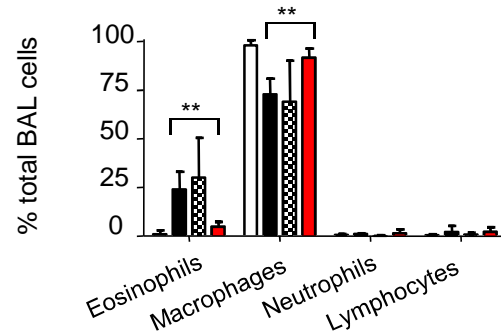
A



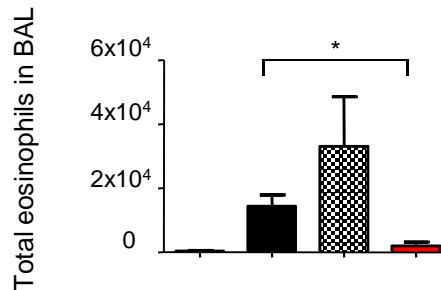
B



C



D



E

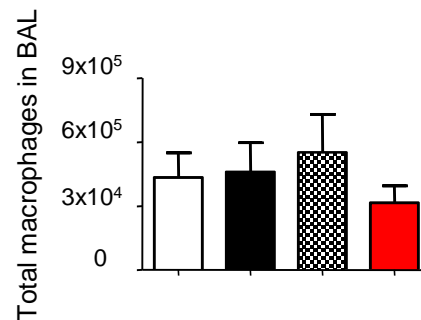


Figure 5.24 2D2 iTreg but not polyclonal iTreg reduce BAL eosinophilia driven by Th2 2D2 cells in rMOG-induced AAI.

FACS sorted GFP⁺ 2D2 or poly iTreg (CD90.1⁺ CD90.2⁺) were injected into WT (CD90.2⁺) host followed by transfer of Th2-polarised 2D2 (CD90.1⁺) cells and 3 consecutive airway challenges with rMOG. Tissue was harvested 2 days after last airway challenge. (A) Experimental scheme. (B) Total cell number in BAL. (C) Percentages of cells in BAL. (D) Total numbers of eosinophils and (E) macrophages. Data are representative of 2-4 experiments, n=4-6. Data are shown as mean \pm SEM. *p<0.05, **p<0.01 as determined by an unpaired t-test.

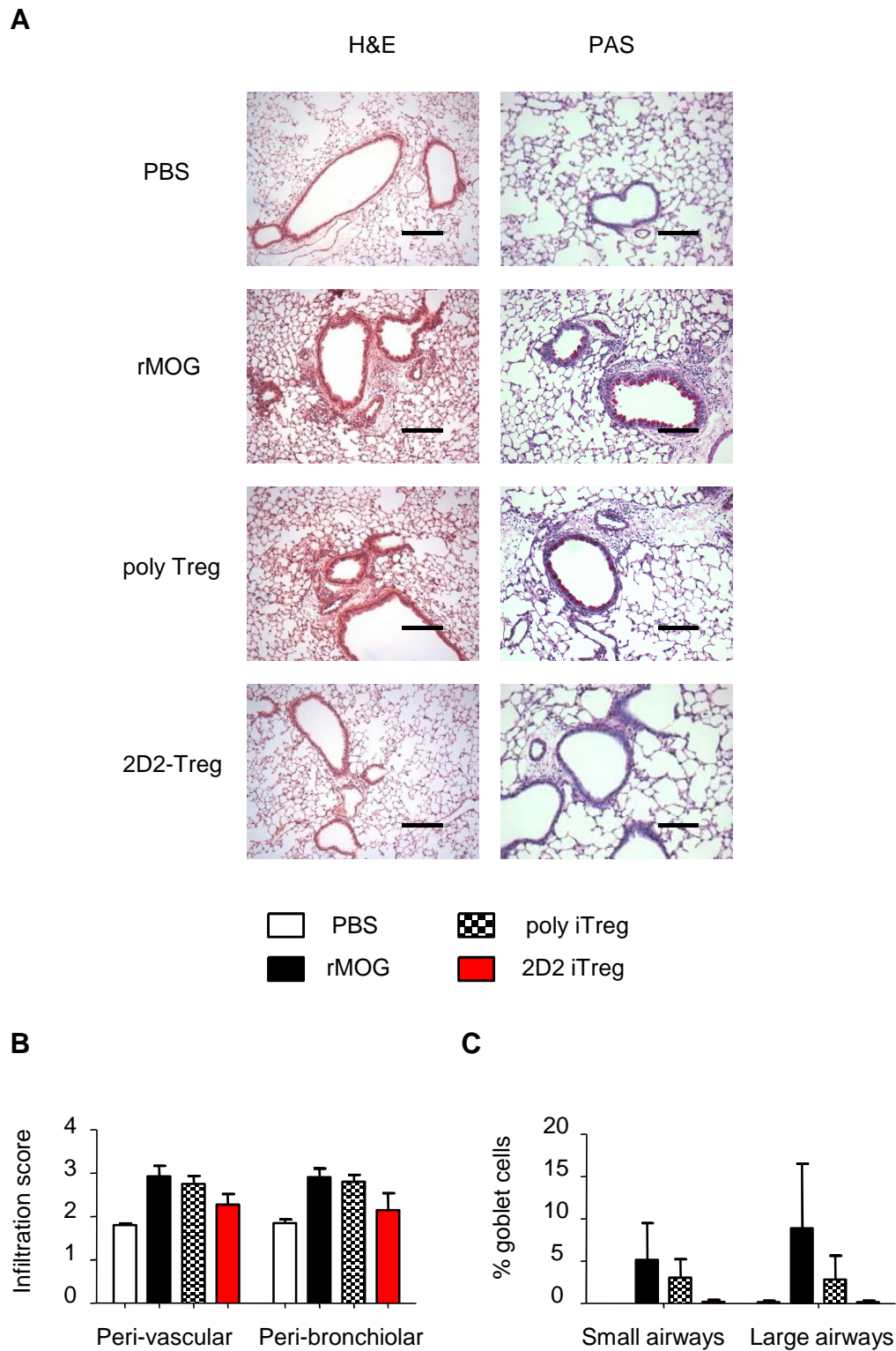


Figure 5.25 2D2 iTreg reduce lung infiltration and goblet cell hyperplasia in rMOG-induced AAI.

Experimental scheme is shown in Figure 5.24. A) H&E and PAS staining (B) Infiltration scores and (C) percentages of goblet cells in the lung. Black line represents 100 μ m. Data representative of 2-4 experiments. Data are shown as mean \pm SEM.

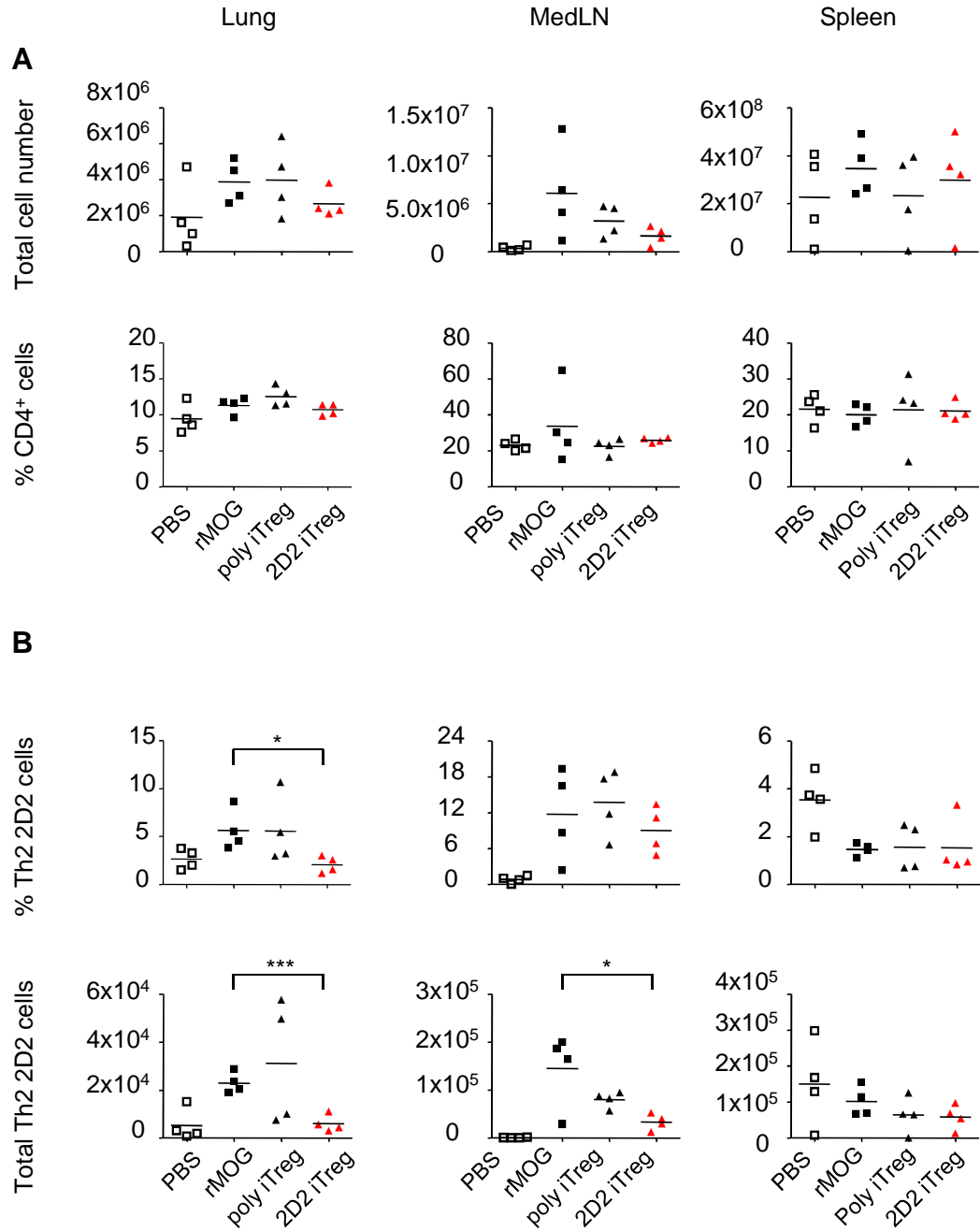


Figure 5.26 2D2 iTreg reduce the numbers of Th2 2D2 cells within the lung and medLN.

Experimental scheme is shown in Figure 5.24. A) Total cell numbers and percentages of CD4⁺ cells. B) Total percentages and numbers of 2D2 Th2 cells. Data are representative of 2-4 experiments, n=4-6. *p<0.05, ***p<0.01 as determined by an unpaired t-test.

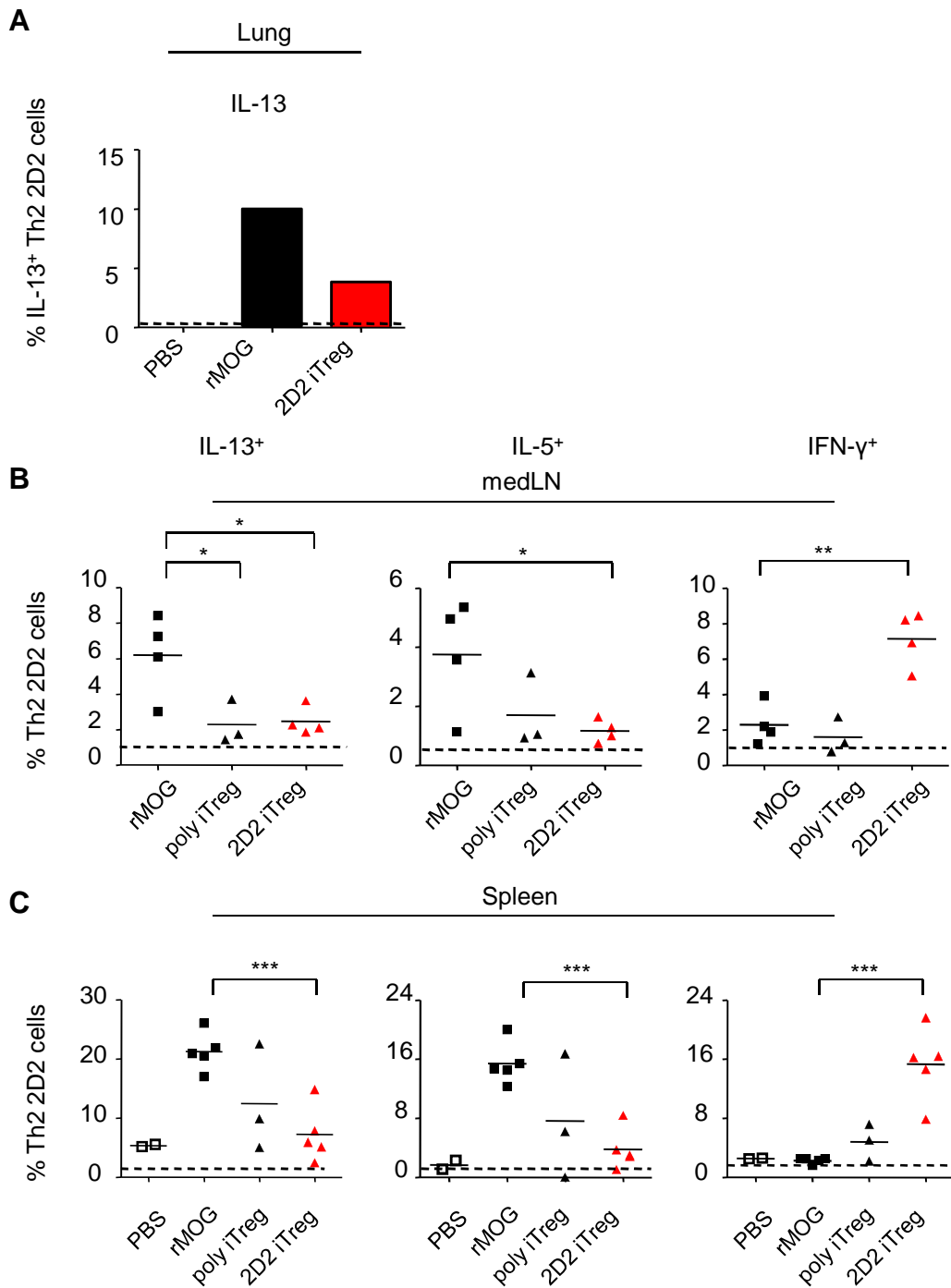


Figure 5.27 2D2 iTreg suppress the production of Th2-associated cytokines and increase the production of IFN- γ .

Experimental scheme is shown in Figure 5.24. Cells from the lung, medLN and spleen were cultured overnight with or without MOG₃₅₋₅₅ followed by ICS staining for cytokines. A) Percentage of IL-13⁺ Th2 2D2 cells (of all Th2 2D2 cells) in the lung, poly iTreg not included, data pooled from 4 mice due to insufficient cell numbers. B) Percentages of IL-13⁺, IL-5⁺ and IFN- γ ⁺ Th2 2D2 cells (of all Th2 2D2 cells) in the medLN and spleen. Dashed lines mark unstimulated control. Data are from (A) one experiment or (B) are representative of 2 experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as determined by an unpaired t-test.

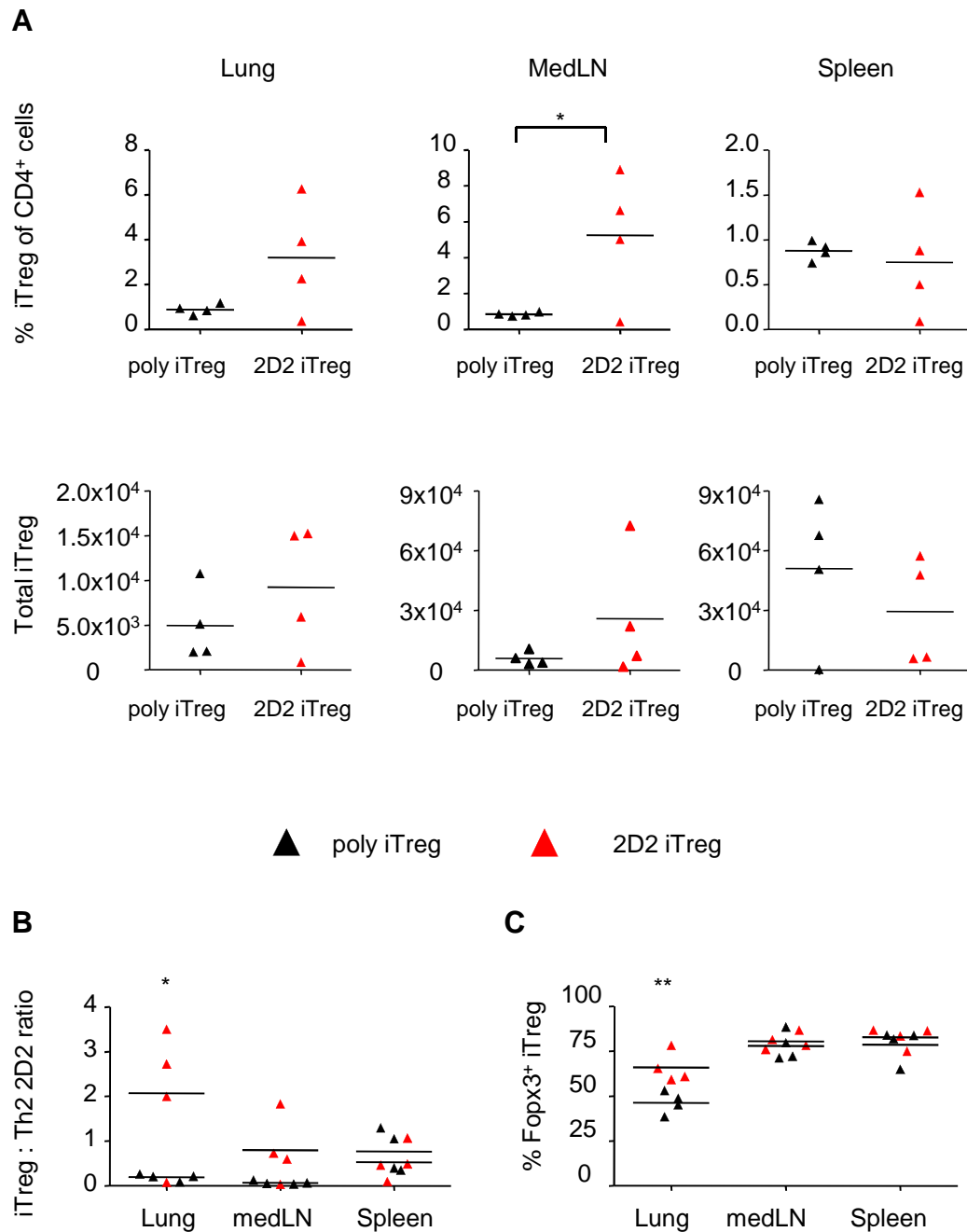
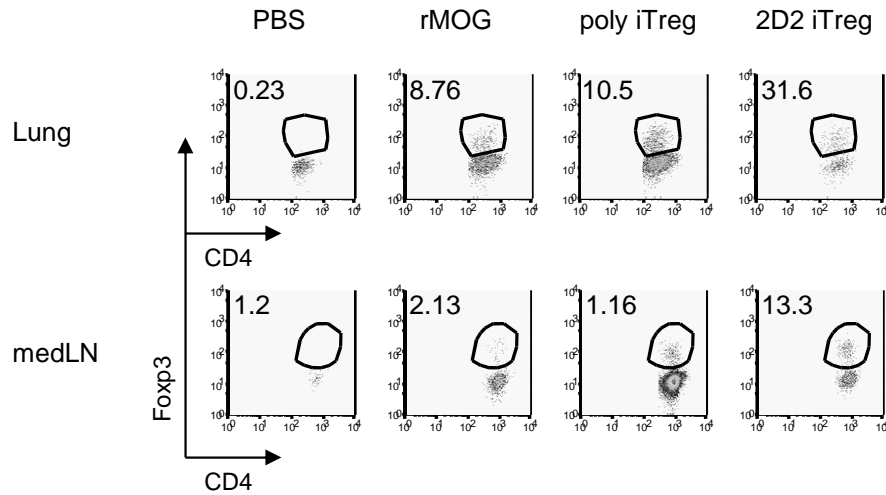


Figure 5.28 The ratio of 2D2 iTreg to Th2 cells is the highest in the lung.

Experimental scheme is shown in Figure 5.24. (A) Percentages and total numbers of iTreg of all CD4⁺ cells. (B) iTreg to Th2 2D2 cell ratio. (C) Percentages of Foxp3⁺ iTreg (of all iTreg). Data are representative of 2-4 experiments. * $p < 0.05$, ** $p < 0.01$ as determined by an unpaired t-test between poly iTreg and 2D2 iTreg.

A



B

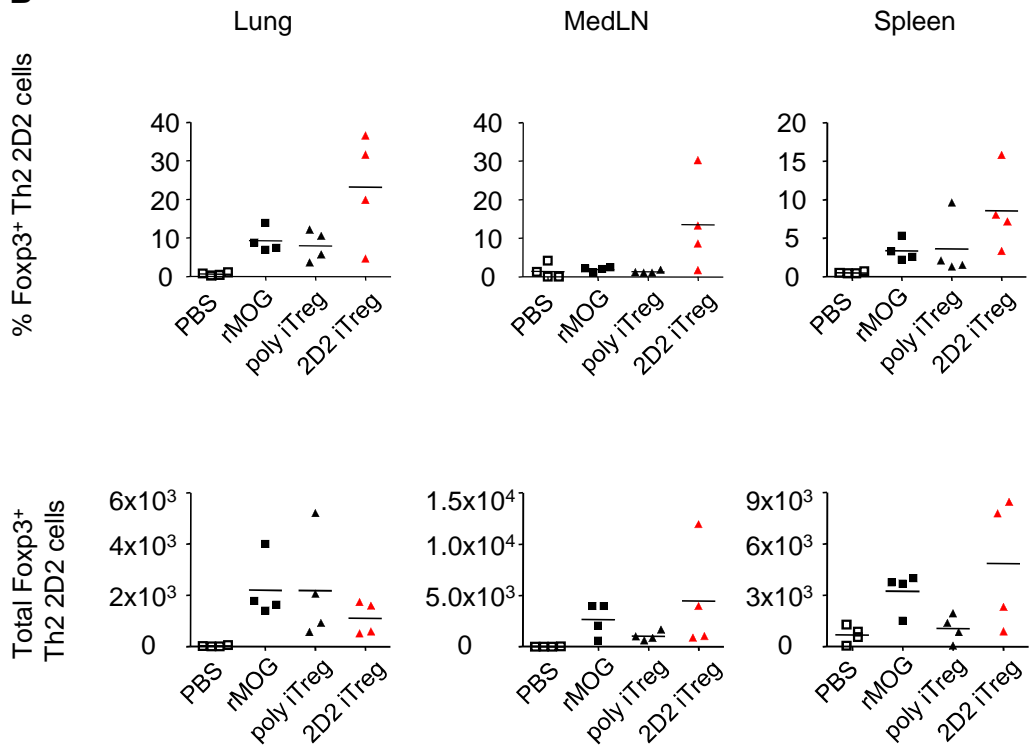


Figure 5.29 2D2 iTreg but not polyclonal iTreg increase the percentages of Foxp3⁺ cells within transferred Th2 2D2 cells.

Experimental scheme is shown in Figure 5.24. (A) Representative flow cytometry plots showing Foxp3 expression in Th2 2D2 cells. (B) Percentages and total numbers of Foxp3⁺ Th2 2D2 cells (% from all Th2 2D2 cells).

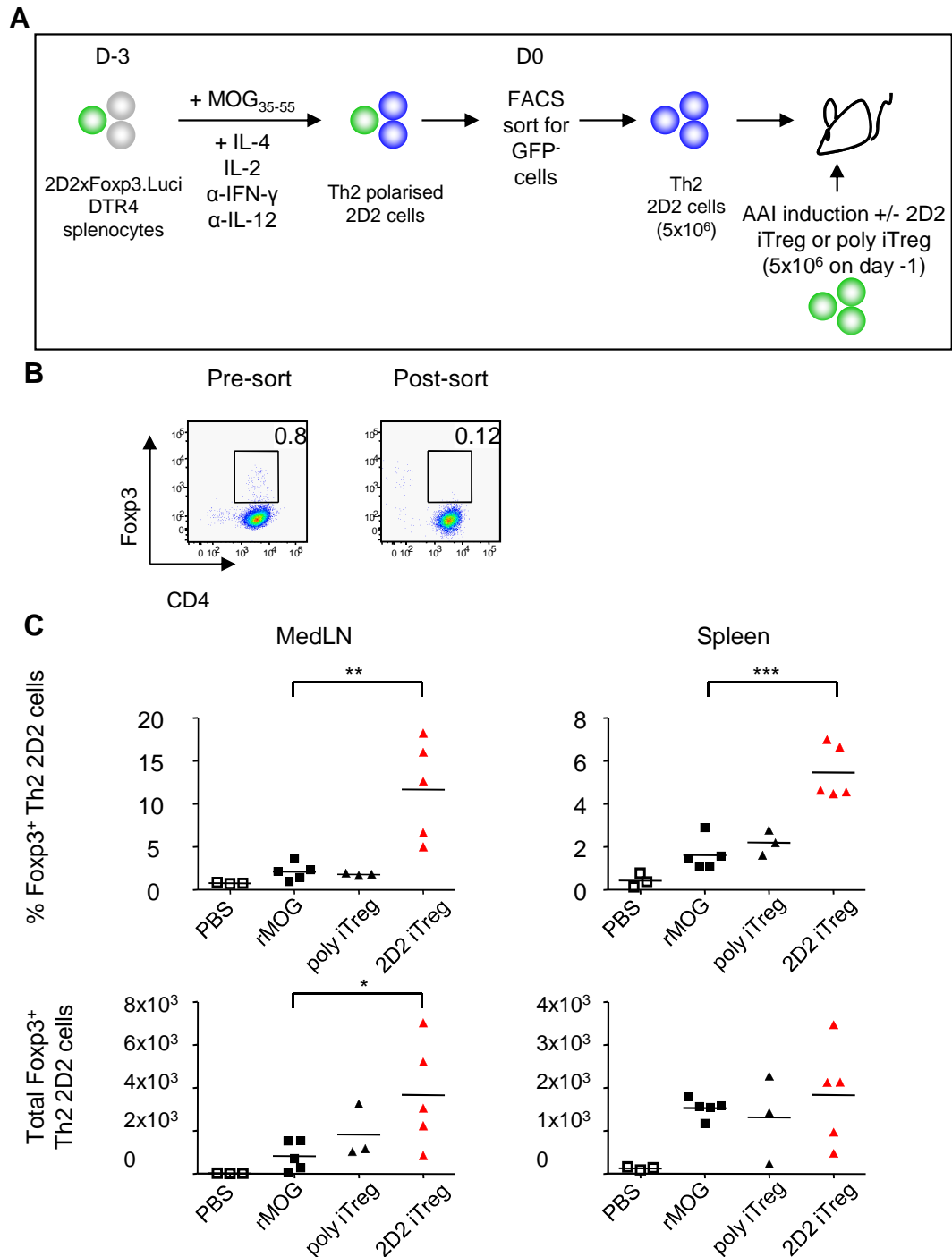


Figure 5.30 2D2 iTreg but not polyclonal iTreg induce expression of Foxp3 in transferred Th2 2D2 cells.

Splenocytes from 2D2xFoxp3.LuciDTR4 (CD90.1⁺ CD90.2⁺) mice were stimulated with MOG₃₅₋₅₅ under Th2 polarising conditions, and FACS sorted for CD4⁺ GFP⁺ cells prior to transfer into WT (CD90.2⁺) mice. FACS-sorted GFP⁺ 2D2 iTreg or polyclonal iTreg (CD90.1⁺) were transferred the day before. AAI was induced by three intratracheal challenges with rMOG. (A) Experimental scheme. (B) Flow cytometry plots showing Foxp3 expression in Th2 2D2 cells pre- and post-sort. (C) Percentages and total numbers of Foxp3⁺ Th2 2D2 cells (from all Th2 2D2 cells). *p<0.05, **p<0.01, ***p<0.001 as determined by an unpaired t-test.

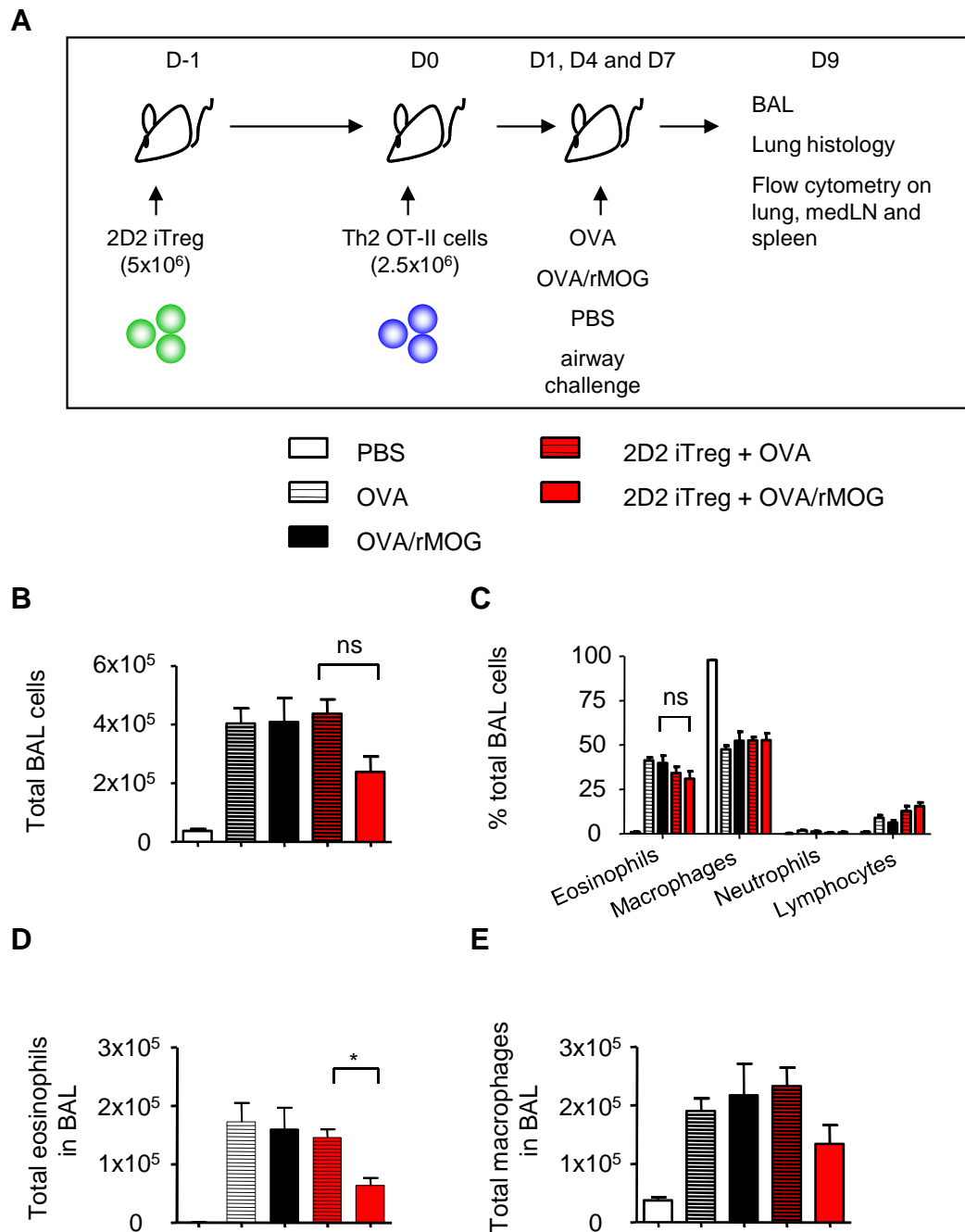


Figure 5.31 2D2 iTreg reduce BAL eosinophilia driven by OVA-reactive Th2 CD4⁺ cells when iTreg cognate antigen is present.

2D2 iTreg (5×10^6) generated from 2D2 (CD90.1⁺ CD45.2⁺) mouse were injected into WT host (CD90.2⁺ CD45.2⁺) followed by transfer of 2.5×10^6 Th2-polarised OT-II CD4⁺ (CD90.2⁺ CD45.1⁺) cells and 3 consecutive airway challenges with OVA, OVA/rMOG or PBS as a control. Tissue was harvested 2 days after last airway challenge. (A) Experimental scheme. (B) Total cell numbers in BAL. (C) Percentages of cells in BAL. (D) Total numbers of eosinophils and (E) macrophages. Data are from one experiment, $n=5-7$. Data are shown as mean \pm SEM. * $p < 0.05$ as determined by an unpaired t-test.

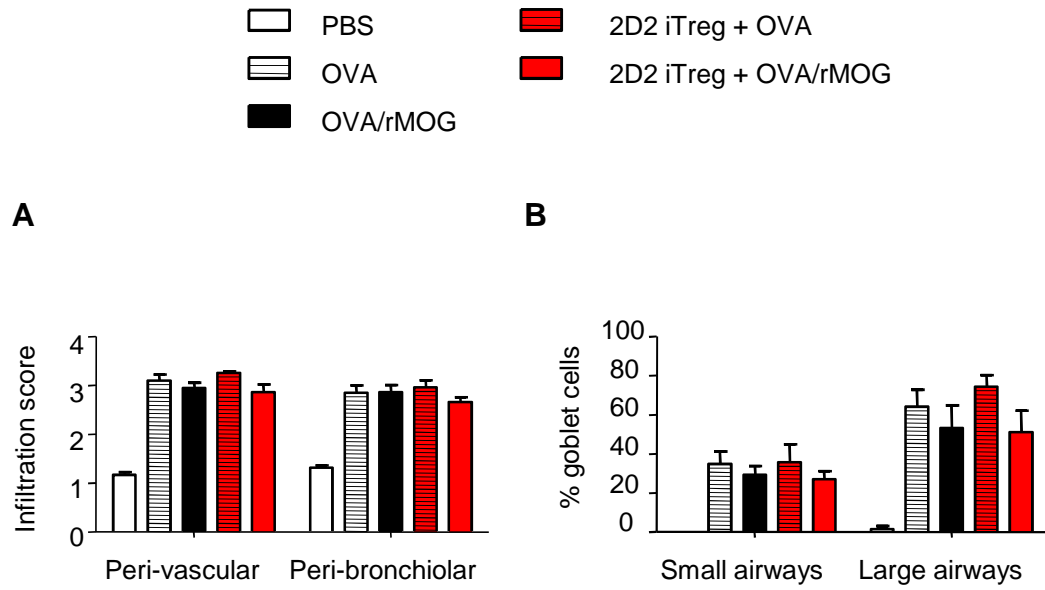


Figure 5.32 2D2 iTreg do not suppress lung infiltration and goblet cell hyperplasia driven by Th2 OT-II cells.

Experimental scheme is shown in Figure 5.31. (A) Infiltration scores and (B) percentages of goblet cells in the lung. Data are from one experiment, n=5-7. Data are shown as mean \pm SEM.

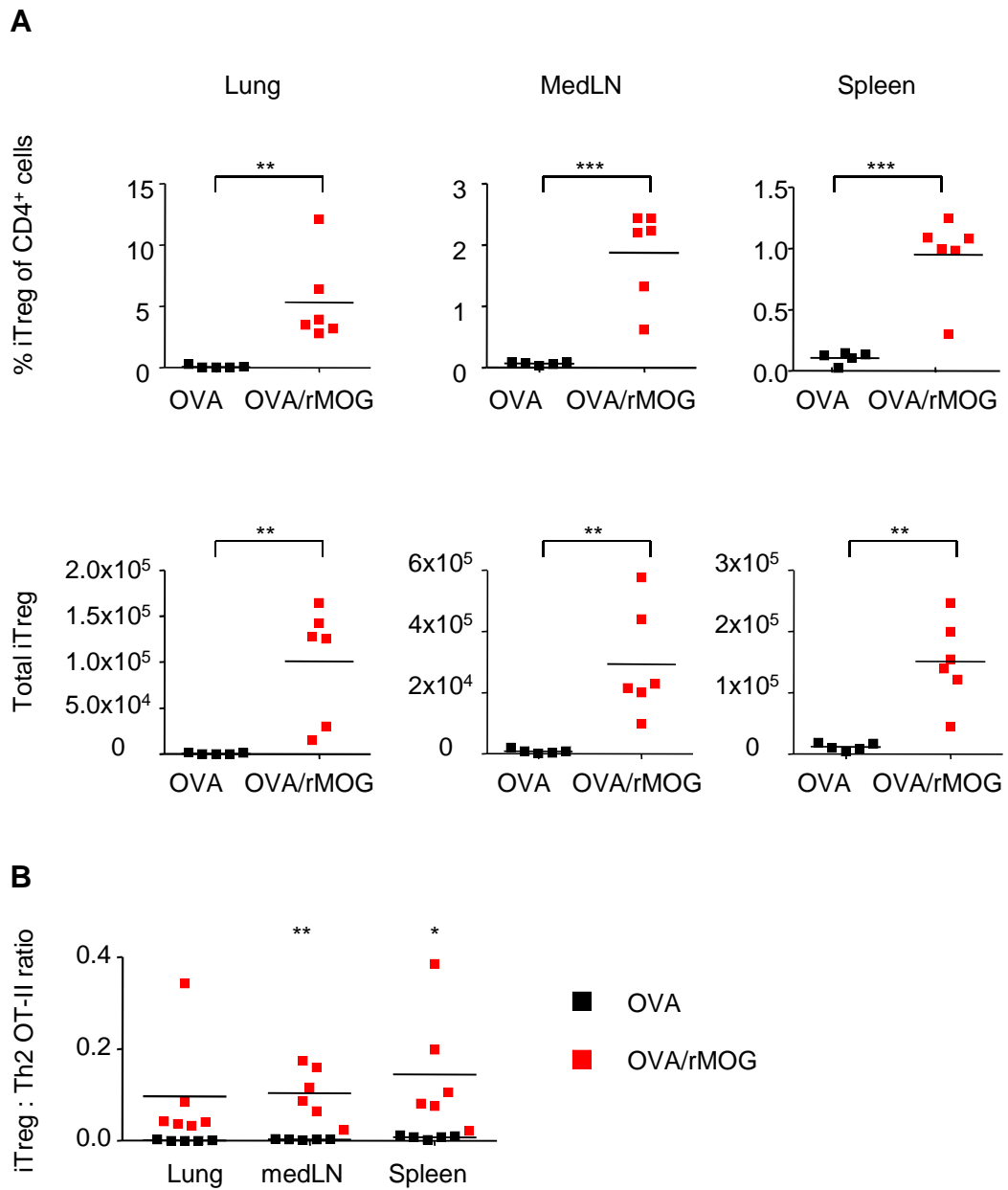


Figure 5.33 The presence of the cognate antigen is critical for 2D2 iTreg migration into the lung and medLN.

Experimental scheme is shown in Figure 5.31. (A) Percentages and total numbers of iTreg. (B) Ratios of iTreg to Th2 OT-II cells. Data are from one experiment, n=5-7. *p<0.05, **p<0.01, ***p<0.001 as determined by an unpaired t-test.

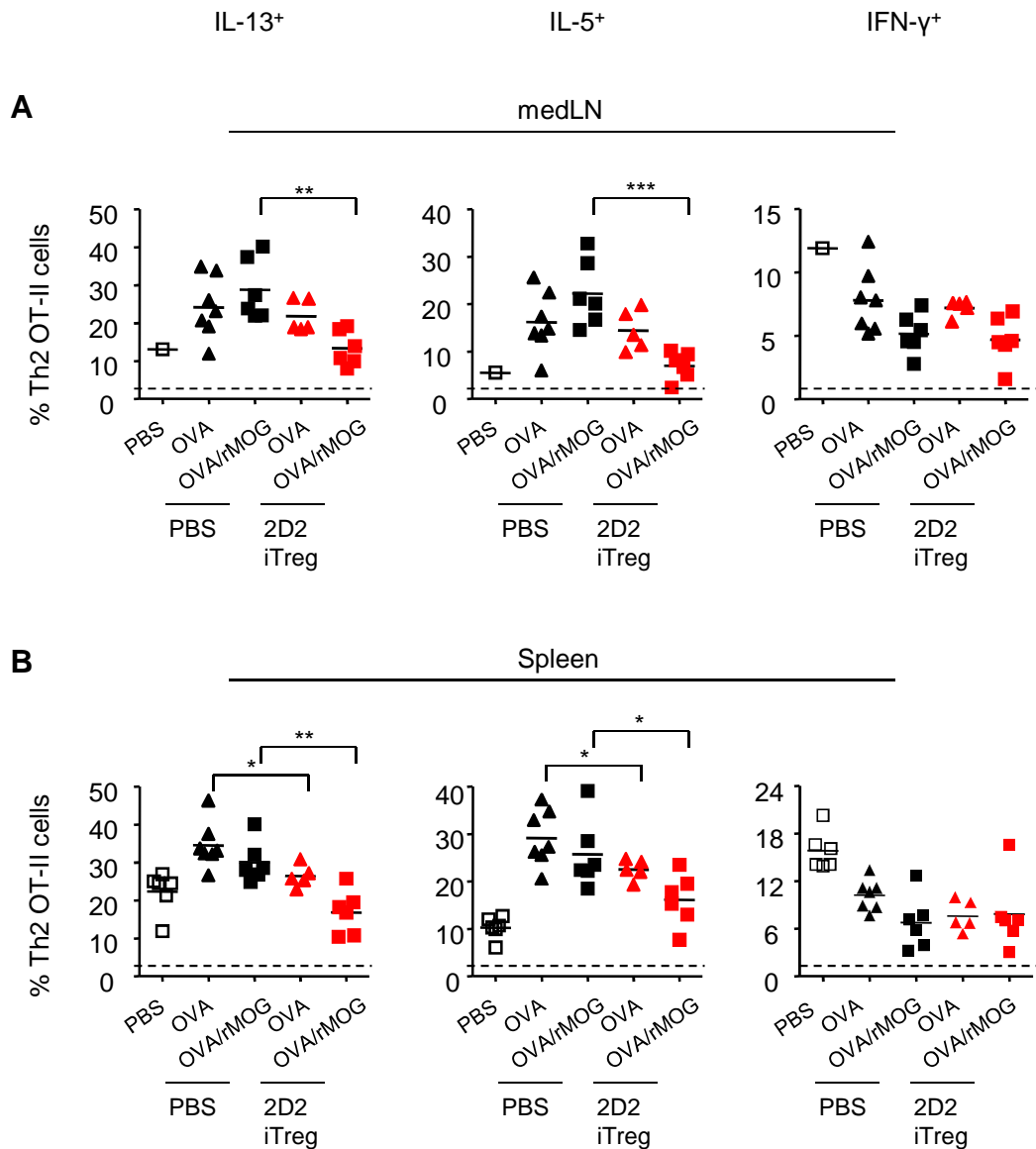


Figure 5.34 2D2 iTreg suppress the production of Th2 OT-II-derived IL-5 and IL-13.

Experimental scheme is shown in Figure 5.31. Cells from the medLN and spleen were cultured overnight with or without OVA₂₂₃₋₂₃₉ and MOG₃₅₋₅₅ followed by ICS staining for cytokines. (A) Percentages of IL-13⁺, IL-5⁺ and IFN- γ ⁺ Th2 OT-II cells in the medLN and (B) spleen. PBS control for medLN was pooled. Dashed lines mark unstimulated control. Data are from one experiment. *p<0.05, **p<0.01, ***p<0.001 as determined by an unpaired t-test.

5.3. Discussion

5.3.1. Effect of Treg phenotype on suppression

It has been suggested that nTreg may be capable of utilizing certain components of Th-associated transcriptional machinery to efficiently control the corresponding type of the immune response (Koch et al., 2009, Zheng et al., 2009). However, the efficiency of polarised nTreg has not been tested against different inflammatory contexts. Due to their poor survival giving insufficient cell numbers, the functional consequences of T-bet and GATA-3 upregulation in nTreg stimulated under Th-polarising conditions *in vitro* could not be tested here. An alternative approach involved expanding naïve nTreg under Th1 or Th2 polarising conditions *in vitro*, as described in Chapter 3, which enabled obtaining sufficient cell numbers for functional analysis. Increased levels of T-bet and CXCR3 in nTreg expanded under Th1 conditions did not affect nTreg ability to suppress Th1 or Th2 cell responses. This suggests that nTreg polarisation may not be important for direct suppressive function *in vitro* but it could be crucial for Treg migration and homeostasis under inflammatory conditions *in vivo*. This is supported by the data that T-bet-deficient Treg retain their suppressive function *in vitro* (Bettelli et al., 2004). However, it has to be noted that that study used naïve CD4⁺ Tresp and not pre-activated Th1 cells. This data also indicates that the expression of T-bet by nTreg does not compromise their suppressive capacity which is consistent with previous reports (Koch et al., 2009). Whether upregulation of GATA-3 under Th2 polarising conditions influences the suppressive function of nTreg *in vitro* needs to be established.

In order to test whether nTreg derived from Th1/Th17 inflammation can suppress Th2-driven inflammation *in vivo*, T-bet⁺ CXCR3^{high} nTreg were isolated from EAE CNS and transferred into WT host prior to AAI induction with the same antigen. These nTreg, previously shown to suppress EAE (McGeachy et al., 2005), were not capable of inhibiting lung inflammation driven by pre-activated Th2 cells even though their numbers were 6-fold higher compared to these used in the EAE study done by McGeachy et al. However, a solid conclusion could not be reached, given alternative possibilities such as poor survival of transferred CNS-derived nTreg or insufficient cell numbers needed for effective suppression of pre-activated Th2 cells.

In the EAE study done by McGeachy et al. CNS-derived nTreg were used to inhibit EAE induction (McGeachy et al., 2005) and there is limited evidence that Treg can inhibit a passive model of EAE driven by a transfer of pre-activated Th1 cells. Moreover, *in vitro* suppression assays presented here showed that pre-activated Th-polarised cells are more resistant to nTreg-mediated suppression compared to naïve cells. Taking into account that the ratio of transferred CNS-derived Treg to Th2 cells was 1:40, and that the frequency of MOG-reactive Treg isolated from the CNS at the peak of EAE is ~7.6% of total Treg (Korn et al., 2007), it is possible that the number of transferred antigen-reactive Treg was too low to observe effective suppression. Due to technical difficulties of this experimental design, a different approach has to be taken to establish how important nTreg phenotype is in suppression of different forms of inflammation and whether it is adaptable. This is further discussed in Chapter 6.

The low numbers of nTreg available for *in vivo* transfer experiments prompted the investigation of the suppressive function of iTreg which can be generated *in vitro* in robust numbers. The default Th1-like iTreg phenotype, in contrast to nTreg, could not be skewed towards being more Th1-like (with higher T-bet expression) or Th2-like (with upregulated GATA-3) by polarising cytokines, unless iTreg stability was compromised. This could mean that a certain level of T-bet and CXCR3 expression could be important for iTreg induction and maintenance of Foxp3 expression.

T-bet⁺ CXCR3⁺ iTreg effectively suppressed both the proliferation and cytokine production by Th1, Th17 and Th2 cells, but were less effective against highly polarised GM-CSF⁺ cells *in vitro*. High expression of T-bet and CXCR3 was also not disadvantageous to iTreg during *in vivo* suppression where they effectively suppressed both Th1/Th17- and Th2-mediated tissue inflammation. The ability of iTreg to inhibit a range of CD4⁺ T cell responses was demonstrated previously in a variety of mouse inflammation models (Huter et al., 2008, Zhang et al., 2010, Xu et al., 2012). This may indicate that nTreg and iTreg use different components of transcriptional machinery to control their suppressive function. Stock et al. demonstrated that iTreg that were induced *in vivo* during a Th1 type of inflammation

expressed T-bet and effectively suppressed Th2-driven inflammation in a sensitization model of AAI (Stock et al., 2004). However, it is unknown whether Th2-like iTreg can be induced *in vivo* from CD4⁺ CD25⁻ cells during Th2 type of inflammation and how effective they are in suppressing Th1 responses. Nevertheless, the Th1-like phenotype of *in vitro* generated iTreg and their ability to suppress a range of CD4⁺ cell responses suggests that iTreg may not have to adapt to the inflammatory environment to effectively regulate the associated Th cell function. Whether iTreg phenotype is modified *in vivo* by inflammation needs to be determined.

5.3.2. Effect of iTreg antigen reactivity on suppression

Suppression of inflammation in EAE and AAI mediated by iTreg was found to be antigen-dependent. 2D2 iTreg effectively suppressed EAE induction whereas polyclonal iTreg failed to exert any effect. This observation is consistent with previous reports showing that only PLP-reactive iTreg suppressed PLP-induced EAE (Zhang et al., 2010) and reflects the data on the importance of antigen-reactivity in nTreg-mediated suppression (Stephens et al., 2009). Since both 2D2 iTreg and polyclonal iTreg were found to suppress naïve CD4⁺ cells *in vitro* but with different efficiencies, it is possible that antigen-driven migration of iTreg to the site of inflammation or the dLN was critical to ensure that sufficient numbers of iTreg accumulated at appropriate site in order to effectively suppress pathogenic CD4⁺ T cell responses. This result disagrees with the previously published efficiency of polyclonal iTreg to inhibit induction of MOG-induced model of EAE (Selvaraj and Geiger, 2008). However, in that study 6x10⁶ of polyclonal iTreg were transferred which is 30 times more than the dose of 2D2 iTreg (2x10⁵ cells) demonstrated to effectively suppress disease severity in this project. Collectively, antigen-reactive iTreg are more effective than polyclonal iTreg and hence can be used at much lower doses.

Although *in vitro* data showed that 2D2 iTreg can inhibit Th1, Th17 and to a lesser extent GM-CSF⁺ cell proliferation and cytokine production, the transfer of 2D2 iTreg

after EAE induction, when the mice showed first clinical signs of a disease, failed to suppress disease severity or to lead to an earlier resolution. 2D2 iTreg were shown to accumulate within the inflamed CNS as the disease progressed and started to resolve. However, they were unable to control on-going inflammation. As GM-CSF produced by effector CD4⁺ cells has been shown to be necessary to drive EAE (Codarri et al., 2011), it is possible that substantial amounts of GM-CSF within the CNS, combined with a poor ability of iTreg to reduce GM-CSF production (as shown by *in vitro* suppression assays) could contribute to the lack of disease control. Moreover, data on iTreg re-stimulation under strong Th1 polarising conditions *in vitro* showed that they rapidly lose Foxp3 and this could contribute to the lack of iTreg-mediated suppression during on-going inflammation. Nevertheless, it has been shown previously that IL-12-conditioned ex-iTreg can still provide protection against EAE if given before immunization (O'Connor et al., 2010).

No previous study has directly compared the efficiency of antigen-reactive versus polyclonal iTreg in a model of Th2-driven AAI. Studies using standard sensitisation / challenge models of AAI have indicated that polyclonal iTreg can effectively suppress disease when transferred prior to sensitization, antigen challenge or after the first antigen challenge but before the disease is established (Chen et al., 2003, Xu et al., 2012). The data from this project, however, indicate that polyclonal iTreg fail to suppress Th2-mediated AAI and only 2D2 iTreg are able to regulate eosinophilia and lung infiltration. The *in vitro* data which showed that polyclonal iTreg do not suppress Th2-associated IL-4 and IL-13 support the *in vivo* observations. Of note, polyclonal iTreg did reduce the levels of IL-13 *in vivo* within the medLN but had no effect on disease severity. The discrepancy between published observations and results from this project is most likely due to the different modes of AAI induction and the timing and dosage of iTreg transfers. Chen et al. injected iTreg twice, 1×10^6 cells during sensitization phase and 5×10^5 cells prior to airway challenge and so it is likely that those iTreg exerted most of their effect by inhibiting the development of the Th2 response (Chen et al., 2003). Xu et al. used 5×10^6 polyclonal iTreg prior to and after the first antigen challenge (Xu et al., 2012). However, the cells that had to be suppressed were most likely memory Th2 cells, rather than freshly activated Th2

cells as used in this project. This suggests that suppression of activation of memory Th2 cells can be mediated by large numbers of polyclonal iTreg, however suppression of proliferation and cytokine production by already activated Th2 cells can only be effectively mediated by antigen-reactive iTreg. Whether these are able to ameliorate AAI once the lung inflammation has been established needs to be determined, requiring a model with greater chronicity.

5.3.3. How do iTreg suppress?

5.3.3.1. Antigen-specific suppression of EAE induction

Transferred antigen-reactive 2D2 iTreg were demonstrated to proliferate *in vivo* in response to antigen and to migrate to the lymph node draining the site of immunization. When co-transferred with naïve 2D2 responder cells, on day 4 post-immunization they limited 2D2 cell survival and cytokine production but not cell proliferation. In EAE, 2D2 iTreg transferred prior to disease induction significantly reduced CD4⁺ T cell infiltrate within the CNS. Moreover, they also prevented splenic lymphopenia normally observed in EAE mice as a result of cell mobilisation due to on-going inflammation. Interestingly, total cell numbers and numbers of CD4⁺ cells were significantly increased in dLN of mice treated with 2D2 iTreg (at both doses) compared to poly iTreg- and PBS-treated groups. This suggests that the protective effect of 2D2 iTreg could be due to retention of CD4⁺ effector T cells in dLN and hence limited Teff mobilization into the CNS. This is supported by the finding that 2D2 iTreg accumulated within dLN, and indicates that dLN is the main site of 2D2 iTreg action. Similar observations were made in studies by the Shevach group who used 1x10⁶ polyclonal nTreg and iTreg to suppress MOG₃₅₋₅₅-driven induction of EAE (Davidson and Shevach, 2011). They reported that reduced EAE severity corresponded with reduced percentages of Teff within the CNS. Moreover, using polyclonal nTreg and TCR transgenic Foxp3⁻ T cell transfer prior to EAE induction, they showed that nTreg did not limit Foxp3⁻ T cell proliferation but rather retained Teff in lymph nodes draining the site of immunization. Although in this study proliferation of Teff was not measured, increased percentages and total cell numbers of Teff in dLN suggest that a similar phenomenon occurred. The Shevach group also

demonstrated that the higher number of Teff in dLN was associated with the lower Teff numbers in the blood suggesting that nTreg were able to restrain Teff from entering the circulation from the dLN and hence prevent them from migrating to the target organ (Davidson and Shevach, 2011). The unaffected Teff proliferation but reduced migration of Teff into the target organ mediated by antigen-reactive iTreg was also reported in a model of autoimmune gastritis (Nguyen et al., 2011). As the potential mechanisms the Shevach group suggested nTreg-mediated downregulation in CXCR4, Syndecan-4 and S1P1 which are implicated in cell mobility (Longley et al., 1999, Thangada et al., 2010). It would be interesting to know whether the levels of these proteins were affected by antigen-reactive iTreg in this study. Moreover, it would be of great value to determine whether 2D2 iTreg are able to produce chemokines that could affect Teff mobilization and hence contribute to their protective function. Antigen-reactive iTreg were shown to secrete a number of chemokines such as CCL3 and CCL4 (in a model of autoimmune gastritis) when activated in inflammatory environment and these were found to be distinct from chemokines produced by nTreg and Teff (Nguyen et al., 2011). These chemokines were found to preferentially induce migration of iTreg themselves into the target organ and reduce the migration of Teff.

Even though negligible numbers of 2D2 iTreg were present in the CNS itself on day 14 of the EAE, this may be associated with a late time point of the harvest. It is possible that 2D2 iTreg migrated into the CNS early after injection and prevented activated CD4⁺ cells from entering the CNS or reducing their effector function and/or survival within the CNS itself. 2D2 iTreg can efficiently migrate to the target organ in response to their cognate antigen even in the absence of inflammation as shown in the lung with a single intratracheal antigen challenge. As the BBB is impermeable to solutes and cells in healthy mice, the migration into the CNS could be facilitated by PTx which was shown to increase permeability of the BBB (Linthicum et al., 1982) and induce leukocyte recruitment into the CNS (Kerfoot et al., 2004). In order to determine the migratory potential of 2D2 iTreg, early time-points after cell transfer would have to be investigated.

In addition to decreased CD4⁺ effector cell numbers within the CNS in 2D2 iTreg-treated mice, the production of pro-inflammatory IFN- γ , TNF- α and GM-CSF, but not IL-17A, was significantly reduced as well. This is consistent with the lack of inflammatory reaction in the CNS and demonstrates that 2D2 iTreg were able to affect not only the total numbers of Teff within target organs but also their effector functions. Similar observations were made with antigen-reactive iTreg in a model of autoimmune gastritis where they were shown to reduce levels of IFN- γ and IL-17 but increase anti-inflammatory IL-10 (Nguyen et al., 2011). Interestingly, the Shevach group did not find evidence of decreased IFN- γ and IL-17 in their study upon polyclonal nTreg/iTreg treatment in EAE and attributed Treg protective effects to suppressed Teff migration only (Davidson and Shevach, 2011). It is possible that iTreg have to be antigen-reactive in order to effectively suppress effector functions of Teff. The production of IFN- γ , TNF- α and GM-CSF was also lower in dLN of 2D2 iTreg-treated mice but unchanged in the spleen and this could be due to the lower percentage of 2D2 iTreg in the spleen versus dLN.

To conclude, the significantly diminished Teff function within the CNS of 2D2 iTreg-treated mice could be due to 2D2 iTreg suppressing the development of inflammatory reaction within the target organ, or inhibiting CD4⁺ cell effector function and migratory capacity in dLN. The 2D2 iTreg accumulation within dLN is in favour of the latter explanation, however this would have to be determined. Of note, the resistance of IL-17 production to Treg-mediated suppression in EAE was reported before and was shown to occur during *in vitro* suppression assay of CNS-derived nTreg and CD4⁺ effector cells (O'Connor et al., 2007). Although 2D2 iTreg effectively suppressed EAE induction, they failed to control on-going CNS inflammation when transferred after disease onset. This could be due to their inability to either regulate already established inflammation itself or to suppress activated Th1/Th17 cells driving the response. Interestingly, 2D2 iTreg were demonstrated to effectively inhibit pre-activated Th2 cells in a model of AAI.

5.3.3.2. Antigen-specific suppression of Th2-driven AAI

2D2 iTreg effectively migrated into the lung and medLN upon antigen airway challenge and suppressed eosinophilia, goblet cell hyperplasia and lung infiltration in a model of AAI driven by pre-activated Th2 cells. This was associated with a significantly decreased representation of transferred Th2 2D2 effector cells within the lung and medLN. Their effector function (production of IL-13 and IL-5) was also reduced within the lung, medLN and spleen. As IL-13 is implicated in the induction of goblet cell hyperplasia (Yang et al., 2001) and IL-5 in the recruitment of eosinophils (Foster et al., 1996), their suppressed levels correlate with the improved disease. Interestingly, 2D2 iTreg increased the percentage of IFN- γ^+ Th2 2D2 cells. However, it is not known whether this corresponds to increased overall levels of IFN- γ within the lung/medLN and hence could contribute to the protective effect of iTreg. IFN- γ inhibits proliferation of Th2 cells (Oriss et al., 1997) and its elevated levels were detected (together with IL-10 and TGF- β) in BAL upon iTreg treatment in a model of cockroach-induced AAI (McGee and Agrawal, 2009). The levels of IFN- γ would have to be tested in the lung tissue/dLN or in BALF in this model.

Although the percentages and absolute numbers of 2D2 iTreg were higher in medLN compared to the lung, the highest iTreg to Th2 2D2 cell ratio (~2) was found in the lung tissue itself. As the total numbers of Th2 2D2 effector cells and their cytokine production was suppressed in both the lung and medLN, this suggests that 2D2 iTreg may exert their suppressive capacity at both sites. It also suggests that 2D2 iTreg may induce cell death of Th2 2D2 effector cells in the lung and medLN but may also limit the migration of Th2 2D2 cells into the lung. The regulation of Teff/iTreg migration mediated by 2D2 iTreg could be due to the production of chemokines as shown in a model of autoimmune gastritis (Nguyen et al., 2011) or due to downregulated expression of molecules implicated in cell mobility as suggested by the Shevach group (Davidson and Shevach, 2011). However, this needs further investigation. It would be also interesting to know whether the mechanisms of suppression mediated by 2D2 iTreg differ not only in Th1/Th17- versus Th2-mediated inflammation but also in regulation of naïve T cells upon activation (as in EAE induction) versus pre-formed Th2 polarised cells.

5.3.3.3. 'Infectious tolerance' as a mode of iTreg-mediated suppression?

2D2 iTreg were found to increase the percentages and total numbers of Foxp3⁺ cells within the transferred naïve 2D2 cells in the dLN and spleen upon immunization. This indicated that 2D2 iTreg were able to either expand the Foxp3⁺ cell population transferred within 2D2 cells or to induce Foxp3 expression in Foxp3⁻ 2D2 cells. One of the first studies describing a phenomenon of 'infectious tolerance' whereby a tolerance-inducing state is transferred from one cell population onto another was reported by the Waldmann lab (Qin et al., 1993). This finding was later expanded by the same group who showed that this 'infectious tolerance' was dependent on Foxp3⁺ Treg induced *de novo* in mice tolerized to transplants (Cobbold et al., 2004). The observation that the generation of these Foxp3⁺ Treg from naïve cells was mediated by Foxp3⁺ nTreg and was TGF-β-dependent was first reported by the Shevach group (Andersson et al., 2008). In EAE it was previously shown that the cells isolated from the dLN of MOG₃₅₋₅₅ immunized, iTreg-treated mice and subsequently depleted of these iTreg could protect from EAE induction (Selvaraj and Geiger, 2008). This suggested that iTreg were capable of inducing a protective cell population.

In this project the transfer of 2D2 iTreg prior to EAE induction led to the increased number of Foxp3⁺ CD4⁺ cells within dLN compared to PBS- and poly iTreg-treated mice which corresponded with the increased total cellularity of dLN. This could be due to the 2D2 iTreg-mediated retention of total CD4⁺ cells within dLN. However, a possibility of the 2D2 iTreg-mediated accumulation of nTreg within dLN or Foxp3 induction in Foxp3⁻ CD4⁺ cells cannot be excluded. Since 'infectious tolerance' was shown to be mediated by TGF-β expression, it would be of interest to determine whether its levels are affected by 2D2 iTreg treatment in dLN. No changes in the level of Foxp3 expression in poly nTreg- and PBS-treated groups were found by the Shevach group in their EAE study (Davidson and Shevach, 2011) which suggests that such changes may only occur in the presence of antigen-reactive Treg.

Interestingly, 2D2 iTreg but not polyclonal iTreg were found to induce Foxp3 expression in transferred pre-activated Th2 2D2 cells within medLN and spleen in a model of AAI. This phenomenon might also occur in the lung. However, low cell

numbers obtained did not permit a full analysis. Induction of Foxp3 expression in effector cells was previously demonstrated to occur *in vitro* in memory Th2 cells (Kim et al., 2010). Those cells were shown to trans-differentiate into Foxp3⁺ cells when stimulated in the presence of TGF- β , all-trans retinoic acid and rapamycin. The converted Th2 memory Treg downregulated expression of GATA-3, produced very low levels of IL-4, IL-5 and IL-13 and effectively suppressed memory Th2 cells *in vitro*, and AHR and eosinophilia upon transfer in a model of AAI in an antigen-dependent fashion (Kim et al., 2010). Moreover, these cells were very stable and capable of suppressing certain disease parameters in already established AAI. The converted Foxp3⁺ Th2 2D2 cells described here which were induced *in vivo* by 2D2 iTreg via ‘infectious tolerance’ could play a role in iTreg-mediated suppression of AAI. The phenotype and functional importance of these converted cells would have to be established.

5.3.3.4. Bystander suppression in a model of AAI

Treg were reported to be able to suppress CD4⁺ cells reactive to a different antigen *in vitro* once they become activated (Thornton and Shevach, 2000). The so-called bystander suppression was also demonstrated *in vivo* in a model of arthritis (Zonneveld-Huijssoon et al., 2011). However, Zhang et al was unable to replicate this observation in a model of PLP-driven EAE (Zhang et al., 2010) posing a question whether phenomenon of Treg-mediated bystander suppression applies to different forms/models of inflammation. A new model of rMOG-induced AAI established in this project and an already established model of Th2 OT-II-driven AAI enabled testing whether 2D2 iTreg were capable of bystander suppression in a Th2 inflammatory context. 2D2 iTreg suppressed Th2 OT-II-driven BAL eosinophilia and Th2-associated cytokines but did not reduce lung infiltration or goblet cell hyperplasia. Moreover, in order to mediate suppression, 2D2 iTreg required the presence of their cognate antigen. One of the reasons for this could be antigen-driven migration of iTreg to the lung and medLN as in its absence negligible numbers of 2D2 iTreg were detected. Another reason could be that iTreg had to be re-activated *in vivo* by their antigen. Suppression of certain but not all disease parameters may be

due to local production of anti-inflammatory cytokines by iTreg upon re-stimulation. IL-10 was suggested by previous studies to mediate protective effects of iTreg (McGee and Agrawal, 2009, Xu et al., 2012). However, in this project *in vitro* re-stimulated iTreg were found to secrete very low levels of IL-10 and high levels of IFN- γ , GM-CSF and IL-2 (Richard O'Connor, personal communication, unpublished data). It is possible that TGF- β is implicated and its production by 2D2 iTreg would have to be assessed.

The lack of inhibition of lung infiltration and goblet cell hyperplasia could be due to a large difference in disease severity between the rMOG-induced and the OVA-induced AAI models. 2D2 iTreg might be able to effectively suppress a moderate degree of inflammation in the rMOG model but not the substantial inflammation in the OVA model. An alternative explanation would be that effective iTreg-mediated suppression *in vivo* is contact-dependent and this requires iTreg and CD4⁺ T effector cells to be of the same antigen-reactivity. This could also explain the lack of suppression mediated by polyclonal iTreg in addition to their reduced antigen-driven migratory capacity.

5.3.4. Stability of iTreg in inflamed environment

Rapid loss of Foxp3 in re-stimulated iTreg in the absence of TGF- β (Floess et al., 2007) led to the concerns over their potential to convert into effector T cells and hence contribute to the inflammation when used in a therapeutic setting. Moreover, a number of studies reported on Treg instability under inflammatory conditions rendering them either ineffective or even pathogenic (Peng et al., 2005, Zhou et al., 2009b, Beres et al., 2011). It is unclear what exactly drives the loss of Foxp3 expression in iTreg *in vivo* and under what conditions this compromises their regulatory function in different disease settings.

In this study, iTreg were demonstrated to limit survival and cytokine production of the co-transferred TCR transgenic CD4⁺ cells (Tresp) upon immunization. This was achieved irrespective of the loss of Foxp3 expression in ~70% of iTreg in dLN and

~40-50% of iTreg in the spleen from a starting population of ~85% Foxp3⁺ iTreg. Interestingly, the loss of Foxp3 was similar irrespective of Tresp co-transfer indicating that in this case it is the antigen-driven iTreg re-stimulation *in vivo* rather than inflammation that leads to the Foxp3 expression instability in iTreg. In EAE, the Foxp3 stability of the protective iTreg transfer prior to disease induction could not be determined due to insufficient cell numbers on the day of harvest. However, when iTreg were transferred after disease induction and analysed 14 days after, ~40% of 2D2 iTreg within the CNS and ~70% in the dLN/spleen retained their Foxp3 expression. Moreover, the similar loss of Foxp3 expression by polyclonal iTreg within dLN/spleen suggested that, in the EAE setting, the loss of Foxp3 in iTreg in secondary lymphoid organs is driven by inflammation and not by the presence of iTreg cognate antigen. However, it is important to highlight the fact that 2D2 iTreg were ineffective in this setting even though they retained their Foxp3. Possible reasons for this and implications are further discussed in Chapter 6.

In AAI mediated by pre-activated Th2 cells, the Foxp3 expression was retained by ~65% of 2D2 iTreg and ~ 50% of polyclonal iTreg within the lung compared to ~80% in the medLN and spleen in both groups. The Foxp3 stability of 2D2 iTreg and polyclonal iTreg could be analyzed in the inflamed target organs in this disease setting, and it revealed that 2D2 iTreg were more stable than polyclonal iTreg. This suggested that iTreg cognate antigen may be important for maintaining their Foxp3 expression. Similarly to EAE, no difference between 2D2 iTreg and polyclonal iTreg was noted in the medLN/spleen in AAI, and the highest Foxp3 loss was noted in the target organs itself.

How inflammation could affect Foxp3 stability in iTreg *in vivo* could be due to a number of ways. It has been reported that pro-inflammatory cytokines such as IL-1 β and TNF can downregulate Foxp3 expression in human Treg (Deknuydt et al., 2009, Valencia et al., 2006). Moreover, in this project it has been shown that *in vitro* re-activation of iTreg in the presence of IL-12, IL-18 or IL-4 leads to the rapid loss of Foxp3 unless exogenous TGF- β is present. In addition to this, rapidly proliferating

pathogenic T cells need IL-2 for their survival and may compete for it with iTreg which require IL-2 to maintain their expression of Foxp3 (Chen et al., 2011).

These data collectively indicate that the Foxp3 instability in iTreg in a disease setting is due to inflammation rather than antigen-driven re-stimulation *in vivo*. Importantly, even though iTreg partially lose their Foxp3 *in vivo*, they remain suppressive. However, it has to be remembered that this is achieved prior to the establishment of inflammation. Retention of Foxp3 in majority of iTreg (~75%) during on-going inflammation was reported by Nguyen et al. suggesting that iTreg have a potential to be stable under already established inflammatory conditions as well (Nguyen et al., 2011).

5.4. Concluding synopsis

- Upregulation of T-bet and CXCR3 expression by nTreg expanded under Th1 conditions did not affect their ability to suppress Th1 or Th2 responses *in vitro*.
- CNS-derived nTreg (T-bet⁺ CXCR3^{high}) did not suppress Th2-driven lung inflammation in a model of AAI (this needs further verification).
- 2D2 iTreg suppressed naïve cell proliferation *in vitro* more effectively than polyclonal iTreg.
- 2D2 iTreg but not polyclonal iTreg effectively inhibited induction of EAE.
- iTreg accumulated within dLN and reduced the total number of effector cells and cytokine production within the CNS.
- 2D2 iTreg but not polyclonal iTreg suppressed proliferation and cytokine production by pre-activated 2D2 CD4⁺ effector cells *in vitro*.
- 2D2 iTreg were unable to suppress ongoing CNS inflammation in EAE even though they accumulated within the CNS.
- 2D2 iTreg but not polyclonal iTreg effectively suppressed AAI driven by pre-activated 2D2 Th2 cells.
- In AAI, iTreg reduced total numbers of effector cells and effector cytokines within the lung and medLN.
- 2D2 iTreg induced Foxp3 expression in Th2 2D2 effector cells in AAI.

- 2D2 iTreg mediated bystander suppression of BAL eosinophilia and Th2-associated cytokines in the presence of their cognate antigen in a model of Th2 OT-II-driven AAI.
- Foxp3 expression instability in iTreg is associated with inflammation rather than antigen-driven re-stimulation of iTreg *in vivo*. Moreover, the presence of iTreg cognate antigen is associated with better maintenance of Foxp3 expression and/or survival of iTreg *in vivo*.

In a summary, Th1-like iTreg effectively suppressed induction of Th1/Th17-mediated inflammation in EAE and the development of AAI driven by pre-activated Th2 cells in an antigen-dependent manner. In terms of nTreg specialization, more experimental evidence is needed to establish the importance of Treg phenotype for their effective suppressive function.

6 General discussion

Foxp3⁺ regulatory T cells have been shown to be crucial in preventing inflammation in autoimmunity and allergic diseases (McGeachy et al., 2005, Kearley et al., 2005, Kim et al., 2007). Previous reports have suggested that Treg may specialize in different types of inflammation by using Th-associated transcriptional machinery to effectively control the corresponding types of Th cells (Koch et al., 2009, Zheng et al., 2009). This thesis tested the hypothesis that Treg acquire different phenotypes in Th1- versus Th2-mediated inflammatory diseases in-line with their effector CD4⁺ T cell (Teff) counterparts and can therefore optimally specialize to control such Teff responses.

The results can be summarised as follows:

- Treg acquire a Th1-like phenotype (T-bet⁺ GATA-3^{low} CXCR3^{high}) specifically within the inflamed CNS in Th1/Th17-mediated EAE and a Th2-like phenotype (T-bet⁻ GATA3⁺) within the inflamed lung in Th2-mediated AAI.
- IL-12 and IL-18, or IL-4 are sufficient to polarise nTreg *in vitro* towards a Th1- or Th2-like phenotype, respectively.
- CNS-derived Treg from a model of EAE appear to be incapable of suppressing lung inflammation in Th2-driven AAI (this needs verification).
- *In vitro* generated iTreg have a default Th1-like phenotype (T-bet⁺ CXCR3^{high}) which cannot be modified *in vitro* during induction/re-stimulation of iTreg without compromising Foxp3 expression.
- Th1-like iTreg effectively suppress inflammation in a model of Th1/Th17-mediated EAE and in a model of Th2-mediated AAI in an antigen-dependent manner.
- iTreg cognate antigen is critical to their survival/migration to the site of inflammation.
- 2D2 iTreg suppress EAE induction but are unable to control on-going inflammation despite their accumulation within the CNS.

- 2D2 iTreg inhibit rMOG-induced AAI driven by an adoptive transfer of pre-activated Th2 2D2 cells.
- iTreg partially lose Foxp3 expression in the inflamed tissue irrespective of antigen presence. However, they retain Foxp3 expression in secondary lymphoid organs.
- Antigen-reactive but not polyclonal iTreg reduce numbers of Teff and pro-inflammatory cytokines within target organs in EAE and AAI.
- iTreg promote Foxp3 induction and/or migration of Foxp3⁺ cells in EAE specifically within/into dLN and induce Foxp3 expression in transferred Th2 2D2 cells in AAI within secondary lymph organs in an antigen-dependent manner.
- iTreg suppress eosinophilia and production of Th2-associated cytokines in AAI in a bystander fashion. However, they do not limit lung infiltration or goblet cell hyperplasia.

These results demonstrate substantial differences in the phenotype of nTreg and *in vitro* generated iTreg which may reflect how these cells adapt to the inflammatory environment to effectively regulate it. Moreover, they highlight the importance of antigen-reactivity in effective iTreg-mediated suppression.

6.1. The importance of Treg phenotype and antigen reactivity in the control of inflammation

Treg have been suggested to be able to specialize in different types of inflammation to better control the corresponding type of CD4⁺ cells driving the inflammatory response (Koch et al., 2009, Zheng et al., 2009). However, how this specialization affects Treg suppression in different inflammatory settings has not been addressed. A new model of rMOG-induced AAI established in this project enabled testing the importance of Treg phenotype in the suppression of Th1/Th17- versus Th2-mediated inflammatory diseases. Treg derived from the inflamed CNS tissue in EAE expressed T-bet and low levels of GATA-3, whereas Treg from the inflamed lung in AAI lacked T-bet expression and had an upregulated GATA-3 expression. Moreover, they also differentially expressed the Th1-associated chemokine receptor CXCR3 with CNS-derived Treg expressing it at higher level compared to the lung-derived Treg.

As the inflammatory response in both disease models was driven by the same antigen, and it has been showed that antigen-reactive Treg accumulate within inflamed tissue (O'Connor et al., 2007), the transfer of CNS-derived Treg prior to induction of AAI would allow for assessment of the functional consequences of their polarised phenotype. CNS-derived Treg did not suppress Th2-driven inflammation in AAI. However, the result from this technically complicated experiment could not be verified using alternative method.

Nevertheless, the possibility that Treg may need to specialize for their most optimal control of inflammation and may not be easily adaptable towards suppression of different forms of inflammation *in vivo* can have potentially important implications in their use in immunotherapy. First of all, it could mean that naïve Treg currently used in clinical trials (Di Ianni et al., 2011, Brunstein et al., 2011) are not the most efficient suppressive cells and hence therapy may require very large numbers and frequent transfusions of Treg to exert their optimal immunosuppressive effects. Second, it can offer a possibility to instruct Treg phenotype to specifically inhibit certain types of immune responses by targeting highly specialized cells directly into the inflamed tissue.

Interestingly, *in vitro* generated iTreg were found to have a default Th1-like phenotype (T-bet⁺ CXCR3^{high}) and this did not prevent them from effectively suppressing both Th1/Th17-mediated inflammation in EAE and Th2-mediated inflammation in AAI. This suggests that high expression of T-bet itself cannot account for the failure of CNS-derived Treg to inhibit Th2-driven inflammation. It could also mean that nTreg and iTreg may use different intrinsic mechanisms to suppress effector CD4⁺ cell responses with iTreg being effective without the need to adapt to the corresponding type of Teff driving the inflammation.

An important consideration in the efficacious use of iTreg is their antigen reactivity. Multiple studies have suggested that in autoimmune inflammation antigen-reactive iTreg, similarly to nTreg, have superior suppressive abilities when compared to polyclonal iTreg (Stephens et al., 2009, Zhang et al., 2010). In allergic inflammation

this has not been investigated before, but polyclonal nTreg and iTreg were shown to improve the disease (Kearley et al., 2005, Xu et al., 2012). This study has demonstrated that only antigen-reactive iTreg could effectively suppress tissue inflammation in EAE and AAI. iTreg cognate antigen was found to be critical for iTreg survival *in vivo* and their migration to the inflamed tissue. Interestingly, antigen-reactive iTreg in the presence of their cognate antigen suppressed eosinophilia and production of Th2-associated cytokines in AAI driven by Th2 cells responsive to a different antigen. Treg-mediated bystander suppression was previously suggested to be mediated by local IL-10 and TGF- β production (Groux et al., 1997, Roncarolo et al., 2006, Andersson et al., 2008). In this study the observed bystander suppression could be attributed to TGF- β expression as no iTreg-derived IL-10 was detected by ELISA and a cytokine bead array analysis (Richard O'Connor, personal communication, unpublished data). Nevertheless, the lung infiltration scores and goblet cell hyperplasia were unaffected despite the presence of iTreg cognate antigen, suggesting that effective iTreg-mediated suppression *in vivo* may be contact-dependent and this would require iTreg and CD4⁺ T effector cells to be of the same antigen-reactivity. This may have substantial implications in the choice of Treg used for cellular immunotherapy as discussed in the next section.

6.2. Therapeutic application of iTreg in MS and asthma

The question whether suppressive function of Treg can be harnessed for the use in immunotherapy has been addressed by several clinical trials investigating therapeutic potential of naïve or *in vitro* expanded nTreg in graft versus host disease (Di Ianni et al., 2011, Brunstein et al., 2011). However, in chronic inflammatory diseases such as MS and asthma there is evidence that the patient's Treg are defective not only in numbers but also in function (Viglietta et al., 2004, Haas et al., 2005, Hartl et al., 2007). Therefore, expansion of these impaired cells *in vitro* and transfusion into patients, or expansion *in vivo* using low antigen doses as shown in animal models (Apostolou and von Boehmer, 2004, Kretschmer et al., 2005) might prove ineffective in combating inflammation.

In vitro generated iTreg provide an easy means of generating robust numbers of cells from patients naïve CD4⁺ cells and protocols for efficient induction of human iTreg have been described (Lu et al., 2010). However, instability of Foxp3 expression in iTreg and potential conversion into pathogenic cells question the effective use of iTreg in therapy. Nevertheless, multiple studies have indicated that iTreg effectively suppress inflammation in a wide variety of animal models of disease including MS and asthma (Zhang et al., 2010, Xu et al., 2012). Moreover, iTreg re-stimulated under pro-inflammatory conditions were found to lack pathogenic potential and were still able to suppress CNS inflammation in EAE (O'Connor et al., 2010).

The data presented here and previously shown by other studies indicate that antigen-reactive iTreg are much more effective at suppressing inflammation compared to polyclonal iTreg and can be used at low numbers (Stephens et al., 2009, Zhang et al., 2010). This seems to be related to better iTreg survival in the presence of their cognate antigen, efficient migration to the site of inflammation and possible cell contact-dependent interactions between iTreg and Teff. Although in MS, CD4⁺ cells reactive to MBP and PLP have been identified in the peripheral blood and CSF of patients (Illes et al., 1999, Burns et al., 1999, Bielekova et al., 2004), the antigen driving the disease remains elusive and more likely a number of antigens are involved due to epitope spreading as the disease progresses. Epitope spreading is a phenomenon whereby an inflammatory reaction directed at a particular antigen enhances antigen presentation and co-stimulatory abilities of APC and this can result in activation of bystander T cells recognizing different epitopes of the same or a distinct antigen (Vanderlugt and Miller, 2002). This was described to occur in an MBP-induced model of EAE (Lehmann et al., 1993, Perry et al., 1991). Although it is unknown how well CD4⁺ cells derived from peripheral blood and CSF of patients represent pathogenic CD4⁺ cells found within CNS, it is possible that identification of key epitopes driving the immune response in MS will allow generation of a panel of iTreg reactive to different myelin-derived antigens to maximise their efficiency. The issue of antigen-reactivity is easier to overcome in asthma and allergic disease where an allergen is usually well-specified providing a more straightforward approach in generating antigen-reactive iTreg.

Bystander suppression, which is implicated in Treg-mediated suppression of inflammation, describes a phenomenon whereby activated Treg can suppress T cells reactive to different antigens (Groux et al., 1997, Roncarolo et al., 2006, Andersson et al., 2008). This offers a promising tool in therapy when a disease-driving antigen is unknown or multiple antigens could be implicated such as in MS and in patients allergic to multiple antigens. However, the results from this project indicate that iTreg cognate antigen is critical to their survival and migration to the site of inflammation. Moreover, even in the presence of their cognate antigen, iTreg in AAI were able to suppress some Teff responses, but did not improve lung infiltration and goblet cell hyperplasia. This indicates that, to be effectively used in therapy, iTreg have to be antigen reactive and in diseases mediated by several antigens a panel of antigen-reactive iTreg has a better chance to suppress inflammation rather than relying on the effect of bystander suppression.

Another important issue raised by other studies is that Treg may not function optimally in an inflamed environment (Korn et al., 2007). However, it has been shown that Treg drive resolution in models of inflammation such as EAE and AAI (O'Connor et al., 2007, Leech et al., 2007). Sub-optimal function in inflammatory environment, however, may apply to iTreg as they failed to suppress on-going CNS inflammation in EAE. Previous studies have shown that although nTreg can suppress Th1 responses, they are unable to control Th17 responses (O'Connor et al., 2007). In addition to this, here it was observed that 2D2 iTreg effectively suppressed Th1 responses *in vitro* and Th2 responses both *in vitro* and *in vivo*, but they seemed to be unable to regulate highly polarised GM-CSF⁺ cells. As GM-CSF was found to be essential in driving EAE (Codarri et al., 2011), this might explain the lack of control during on-going inflammation when high levels of GM-CSF were detected. These observations suggest that effective cellular therapy involving Treg might have to be combined with additional pharmacological treatment that will reduce on-going inflammation. An example of such treatment could be rapamycin which was shown to inhibit T cells responses to IL-2 simultaneously promoting survival of Treg (Battaglia et al., 2006) and is currently used in organ transplantation.

What also needs to be addressed when considering iTreg as an immunotherapy is the persistence of iTreg *in vivo* and longevity of their suppressive effects. In a model of AAI used in this project, the lung inflammation was short and self-resolving hence iTreg did not need to persist for long and one dose of iTreg was sufficient. 2D2 iTreg were detectable 10 days after the transfer in AAI and approximately 2 weeks after EAE induction. It is unknown how long they would persist in the absence of inflammation and whether they would be able to prevent re-induction of disease in both models. As both MS and asthma are chronic diseases it seems likely that multiple and/or regular treatment would be required.

6.3. Future work

The primary issue that has to be addressed in the future work is the verification whether Th1-like Treg adapted to Th1/Th17-mediated inflammation are truly unable to effectively control Th2-mediated inflammation as indicated in this study. As the alternative approach of using Th1-mediated AAI failed to provide a source of Th1-like Treg, CNS-derived Treg could be perhaps further expanded *in vitro* to increase their numbers for the transfer into AAI model. As already mentioned, it is possible that the frequency of MOG-reactive nTreg within transferred total CNS-derived Treg was insufficient to suppress 5×10^6 of pre-activated 2D2 Th2 effector cells. However, in this case it may be simply unfeasible to sufficiently increase the numbers of Th1-like nTreg from the CNS to inhibit inflammation driven by pre-activated Th2 cells. Different models of inflammation that would provide larger numbers of antigen-reactive Th1-like Treg may have to be considered.

Moreover, in addition to CXCR3, changes in other Th1-associated chemokine receptors such as CCR5 and Th2-associated CCR4 and CCR8 should be tested in nTreg derived from EAE and AAI. It would be of interest to know whether changes in tissue-derived Treg phenotype in EAE and AAI are reflected in epigenetic modifications in the genes that encode upregulated/downregulated transcription factors and chemokine receptors. As epigenetic modifications were shown to be

associated with the stability of gene expression, this would provide additional information on the stability/adaptability of Treg phenotype.

Antigen-reactive iTreg suppressed induction of EAE. However, they were unable to control on-going CNS inflammation. As the regulation of on-going disease and not disease induction is relevant to immunotherapy, it would of great value to know whether iTreg failure to control on-going EAE is associated with their inability to control any form of established inflammation, or an inability to control effector functions of activated T cells (such as GM-CSF production as suggested by *in vitro* studies). This could be determined by an assessment of iTreg protective effect in a passive model of EAE induced by a transfer of lymphocytes polarised under Th1, Th17 or GM-CSF conditions. Since antigen-reactive iTreg effectively suppressed AAI driven by pre-activated Th2 cells it would be interesting to know whether they can regulate an already established Th2-driven inflammation. This would require a chronic model of AAI.

It is also important to determine how long iTreg persist in the target organ and secondary lymphoid organs in EAE and AAI and whether they are able to prevent disease re-induction. The exact mechanism of how 2D2 iTreg are able to restrict Teff access into the CNS in EAE, and how they suppress Teff numbers and effector function in the lung/medLN in AAI should be deciphered, for example, by looking at the expression of chemokines and migratory molecules on Teff/iTreg.

6.4. Closing statement

In a summary, this thesis provides data that indicate substantial phenotypic differences between nTreg and *in vitro* generated iTreg, which may reflect how they are able to control pathogenic CD4⁺ T cells and the resulting inflammatory reaction. Moreover, antigen-reactivity of iTreg was demonstrated to be critical for their effective suppression of autoimmune and allergic inflammation which may have important implications in their use in immunotherapy.

7 References

- Abdul-Majid, K. B., Wefer, J., Stadelmann, C., Stefferl, A., Lassmann, H., Olsson, T. & Harris, R. A. (2003) Comparing the pathogenesis of experimental autoimmune encephalomyelitis in CD4^{-/-} and CD8^{-/-} DBA/1 mice defines qualitative roles of different T cell subsets. *J Neuroimmunol*, 141, 10-9.
- Adamson, A. S., Collins, K., Laurence, A. & O'Shea, J. J. (2009) The Current STATus of lymphocyte signaling: new roles for old players. *Curr Opin Immunol*, 21, 161-6.
- Afkarian, M., Sedy, J. R., Yang, J., Jacobson, N. G., Cereb, N., Yang, S. Y., Murphy, T. L. & Murphy, K. M. (2002) T-bet is a STAT1-induced regulator of IL-12R expression in naive CD4⁺ T cells. *Nat Immunol*, 3, 549-57.
- Aharoni, R., Teitelbaum, D., Arnon, R. & Sela, M. (1999) Copolymer 1 acts against the immunodominant epitope 82-100 of myelin basic protein by T cell receptor antagonism in addition to major histocompatibility complex blocking. *Proc Natl Acad Sci U S A*, 96, 634-9.
- Akbari, O., Freeman, G. J., Meyer, E. H., Greenfield, E. A., Chang, T. T., Sharpe, A. H., Berry, G., DeKruyff, R. H. & Umetsu, D. T. (2002) Antigen-specific regulatory T cells develop via the ICOS-ICOS-ligand pathway and inhibit allergen-induced airway hyperreactivity. *Nat Med*, 8, 1024-32.
- Akdis, M., Verhagen, J., Taylor, A., Karamloo, F., Karagiannidis, C., Cramer, R., Thunberg, S., Deniz, G., Valenta, R., Fiebig, H., Kegel, C., Disch, R., Schmidt-Weber, C. B., Blaser, K. & Akdis, C. A. (2004) Immune responses in healthy and allergic individuals are characterized by a fine balance between allergen-specific T regulatory 1 and T helper 2 cells. *J Exp Med*, 199, 1567-75.
- Akimova, T., Beier, U. H., Wang, L., Levine, M. H. & Hancock, W. W. (2011) Helios expression is a marker of T cell activation and proliferation. *PLoS One*, 6, e24226.
- Al-Ramli, W., Prefontaine, D., Chouiali, F., Martin, J. G., Olivenstein, R., Lemiere, C. & Hamid, Q. (2009) T(H)17-associated cytokines (IL-17A and IL-17F) in severe asthma. *J Allergy Clin Immunol*, 123, 1185-7.
- Anderson, P. O., Manzo, B. A., Sundstedt, A., Minaee, S., Symonds, A., Khalid, S., Rodriguez-Cabezas, M. E., Nicolson, K., Li, S., Wraith, D. C. & Wang, P. (2006) Persistent antigenic stimulation alters the transcription program in T cells, resulting in antigen-specific tolerance. *Eur J Immunol*, 36, 1374-85.
- Andersson, J., Tran, D. Q., Pesu, M., Davidson, T. S., Ramsey, H., O'Shea, J. J. & Shevach, E. M. (2008) CD4⁺ FoxP3⁺ regulatory T cells confer infectious tolerance in a TGF-beta-dependent manner. *J Exp Med*, 205, 1975-81.
- Anderton, S. M., Radu, C. G., Lowrey, P. A., Ward, E. S. & Wraith, D. C. (2001) Negative selection during the peripheral immune response to antigen. *J Exp Med*, 193, 1-11.
- Anderton, S. M. & Wraith, D. C. (2002) Selection and fine-tuning of the autoimmune T-cell repertoire. *Nat Rev Immunol*, 2, 487-98.
- Ando, D. G., Clayton, J., Kono, D., Urban, J. L. & Sercarz, E. E. (1989) Encephalitogenic T cells in the B10.PL model of experimental allergic

- encephalomyelitis (EAE) are of the Th-1 lymphokine subtype. *Cell Immunol*, 124, 132-43.
- Apostolou, I. & von Boehmer, H. (2004) In vivo instruction of suppressor commitment in naive T cells. *J Exp Med*, 199, 1401-8.
- Asano, M., Toda, M., Sakaguchi, N. & Sakaguchi, S. (1996) Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation. *J Exp Med*, 184, 387-96.
- Ascherio, A., Munger, K. L., Lennette, E. T., Spiegelman, D., Hernan, M. A., Olek, M. J., Hankinson, S. E. & Hunter, D. J. (2001) Epstein-Barr virus antibodies and risk of multiple sclerosis: a prospective study. *Jama*, 286, 3083-8.
- Asseman, C., Mauze, S., Leach, M. W., Coffman, R. L. & Powrie, F. (1999) An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. *J Exp Med*, 190, 995-1004.
- Azzawi, M., Bradley, B., Jeffery, P. K., Frew, A. J., Wardlaw, A. J., Knowles, G., Assoufi, B., Collins, J. V., Durham, S. & Kay, A. B. (1990) Identification of activated T lymphocytes and eosinophils in bronchial biopsies in stable atopic asthma. *Am Rev Respir Dis*, 142, 1407-13.
- Bansal-Pakala, P., Jember, A. G. & Croft, M. (2001) Signaling through OX40 (CD134) breaks peripheral T-cell tolerance. *Nat Med*, 7, 907-12.
- Barcellos, L. F., Oksenberg, J. R., Begovich, A. B., Martin, E. R., Schmidt, S., Vittinghoff, E., Goodin, D. S., Pelletier, D., Lincoln, R. R., Bucher, P., Swerdlin, A., Pericak-Vance, M. A., Haines, J. L. & Hauser, S. L. (2003) HLA-DR2 dose effect on susceptibility to multiple sclerosis and influence on disease course. *Am J Hum Genet*, 72, 710-6.
- Barlow, J. L., Bellosi, A., Hardman, C. S., Drynan, L. F., Wong, S. H., Cruickshank, J. P. & McKenzie, A. N. (2012) Innate IL-13-producing nuocytes arise during allergic lung inflammation and contribute to airways hyperreactivity. *J Allergy Clin Immunol*, 129, 191-8 e1-4.
- Barnden, M. J., Allison, J., Heath, W. R. & Carbone, F. R. (1998) Defective TCR expression in transgenic mice constructed using cDNA-based alpha- and beta-chain genes under the control of heterologous regulatory elements. *Immunol Cell Biol*, 76, 34-40.
- Baru, A. M., Hartl, A., Lahl, K., Krishnaswamy, J. K., Fehrenbach, H., Yildirim, A. O., Garn, H., Renz, H., Behrens, G. M. & Sparwasser, T. (2010) Selective depletion of Foxp3⁺ Treg during sensitization phase aggravates experimental allergic airway inflammation. *Eur J Immunol*, 40, 2259-66.
- Battaglia, M., Stabilini, A., Migliavacca, B., Horejs-Hoeck, J., Kaupper, T. & Roncarolo, M. G. (2006) Rapamycin promotes expansion of functional CD4⁺CD25⁺FOXP3⁺ regulatory T cells of both healthy subjects and type 1 diabetic patients. *J Immunol*, 177, 8338-47.
- Bauer, J., Berkenbosch, F., Van Dam, A. M. & Dijkstra, C. D. (1993) Demonstration of interleukin-1 beta in Lewis rat brain during experimental allergic encephalomyelitis by immunocytochemistry at the light and ultrastructural level. *J Neuroimmunol*, 48, 13-21.
- Baxter, A. G. (2007) The origin and application of experimental autoimmune encephalomyelitis. *Nat Rev Immunol*, 7, 904-12.
- Bennett, C. L., Christie, J., Ramsdell, F., Brunkow, M. E., Ferguson, P. J., Whitesell, L., Kelly, T. E., Saulsbury, F. T., Chance, P. F. & Ochs, H. D. (2001) The

- immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat Genet*, 27, 20-1.
- Benveniste, E. N. (1997) Role of macrophages/microglia in multiple sclerosis and experimental allergic encephalomyelitis. *J Mol Med (Berl)*, 75, 165-73.
- Beres, A., Komorowski, R., Mihara, M. & Drobyski, W. R. (2011) Instability of Foxp3 expression limits the ability of induced regulatory T cells to mitigate graft versus host disease. *Clin Cancer Res*, 17, 3969-83.
- Berger, T. (2009) Current therapeutic recommendations in multiple sclerosis. *J Neurol Sci*, 287 Suppl 1, S37-45.
- Besnard, A. G., Togbe, D., Guillou, N., Erard, F., Quesniaux, V. & Ryffel, B. (2011) IL-33-activated dendritic cells are critical for allergic airway inflammation. *Eur J Immunol*, 41, 1675-86.
- Bettelli, E., Korn, T. & Kuchroo, V. K. (2007) Th17: the third member of the effector T cell trilogy. *Curr Opin Immunol*, 19, 652-7.
- Bettelli, E., Pagany, M., Weiner, H. L., Linington, C., Sobel, R. A. & Kuchroo, V. K. (2003) Myelin oligodendrocyte glycoprotein-specific T cell receptor transgenic mice develop spontaneous autoimmune optic neuritis. *J Exp Med*, 197, 1073-81.
- Bettelli, E., Sullivan, B., Szabo, S. J., Sobel, R. A., Glimcher, L. H. & Kuchroo, V. K. (2004) Loss of T-bet, but not STAT1, prevents the development of experimental autoimmune encephalomyelitis. *J Exp Med*, 200, 79-87.
- Bettini, M. L., Pan, F., Bettini, M., Finkelstein, D., Rehg, J. E., Floess, S., Bell, B. D., Ziegler, S. F., Huehn, J., Pardoll, D. M. & Vignali, D. A. (2012) Loss of epigenetic modification driven by the Foxp3 transcription factor leads to regulatory T cell insufficiency. *Immunity*, 36, 717-30.
- Bianchi, M. E. (2007) DAMPs, PAMPs and alarmins: all we need to know about danger. *J Leukoc Biol*, 81, 1-5.
- Bielekova, B., Sung, M. H., Kadom, N., Simon, R., McFarland, H. & Martin, R. (2004) Expansion and functional relevance of high-avidity myelin-specific CD4+ T cells in multiple sclerosis. *J Immunol*, 172, 3893-904.
- Bienvenu, B., Martin, B., Auffray, C., Cordier, C., Becourt, C. & Lucas, B. (2005) Peripheral CD8+CD25+ T lymphocytes from MHC class II-deficient mice exhibit regulatory activity. *J Immunol*, 175, 246-53.
- Billiau, A., Heremans, H., Vandekerckhove, F., Dijkmans, R., Sobis, H., Meulepas, E. & Carton, H. (1988) Enhancement of experimental allergic encephalomyelitis in mice by antibodies against IFN-gamma. *J Immunol*, 140, 1506-10.
- Bochner, B. S. & Gleich, G. J. (2010) What targeting eosinophils has taught us about their role in diseases. *J Allergy Clin Immunol*, 126, 16-25; quiz 26-7.
- Boise, L. H., Minn, A. J., Noel, P. J., June, C. H., Accavitti, M. A., Lindsten, T. & Thompson, C. B. (1995) CD28 costimulation can promote T cell survival by enhancing the expression of Bcl-XL. *Immunity*, 3, 87-98.
- Boissonnas, A., Scholer-Dahirel, A., Simon-Blancal, V., Pace, L., Valet, F., Kissenpfennig, A., Sparwasser, T., Malissen, B., Fetler, L. & Amigorena, S. (2010) Foxp3+ T cells induce perforin-dependent dendritic cell death in tumor-draining lymph nodes. *Immunity*, 32, 266-78.
- Bollyky, P. L., Lord, J. D., Masewicz, S. A., Evanko, S. P., Buckner, J. H., Wight, T. N. & Nepom, G. T. (2007) Cutting edge: high molecular weight hyaluronan

- promotes the suppressive effects of CD4+CD25+ regulatory T cells. *J Immunol*, 179, 744-7.
- Booth, H., Richmond, I., Ward, C., Gardiner, P. V., Harkawat, R. & Walters, E. H. (1995) Effect of high dose inhaled fluticasone propionate on airway inflammation in asthma. *Am J Respir Crit Care Med*, 152, 45-52.
- Borish, L. C., Nelson, H. S., Corren, J., Bensch, G., Busse, W. W., Whitmore, J. B. & Agosti, J. M. (2001) Efficacy of soluble IL-4 receptor for the treatment of adults with asthma. *J Allergy Clin Immunol*, 107, 963-70.
- Bousso, P. (2008) T-cell activation by dendritic cells in the lymph node: lessons from the movies. *Nat Rev Immunol*, 8, 675-84.
- Brennan, F. M., Hayes, A. L., Ciesielski, C. J., Green, P., Foxwell, B. M. & Feldmann, M. (2002) Evidence that rheumatoid arthritis synovial T cells are similar to cytokine-activated T cells: involvement of phosphatidylinositol 3-kinase and nuclear factor kappaB pathways in tumor necrosis factor alpha production in rheumatoid arthritis. *Arthritis Rheum*, 46, 31-41.
- Bretscher, P. & Cohn, M. (1970) A theory of self-nonsel discrimination. *Science*, 169, 1042-9.
- Bright, J. J., Du, C., Coon, M., Sriram, S. & Klaus, S. J. (1998a) Prevention of experimental allergic encephalomyelitis via inhibition of IL-12 signaling and IL-12-mediated Th1 differentiation: an effect of the novel anti-inflammatory drug lisofylline. *J Immunol*, 161, 7015-22.
- Bright, J. J., Musuro, B. F., Du, C. & Sriram, S. (1998b) Expression of IL-12 in CNS and lymphoid organs of mice with experimental allergic encephalitis. *J Neuroimmunol*, 82, 22-30.
- Brinkmann, V., Billich, A., Baumruker, T., Heining, P., Schmouder, R., Francis, G., Aradhye, S. & Burtin, P. (2010) Fingolimod (FTY720): discovery and development of an oral drug to treat multiple sclerosis. *Nat Rev Drug Discov*, 9, 883-97.
- Bruck, W. (2005) The pathology of multiple sclerosis is the result of focal inflammatory demyelination with axonal damage. *J Neurol*, 252 Suppl 5, v3-9.
- Brunkow, M. E., Jeffery, E. W., Hjerrild, K. A., Paeper, B., Clark, L. B., Yasayko, S. A., Wilkinson, J. E., Galas, D., Ziegler, S. F. & Ramsdell, F. (2001) Disruption of a new forkhead/winged-helix protein, scurfy, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat Genet*, 27, 68-73.
- Brunstein, C. G., Miller, J. S., Cao, Q., McKenna, D. H., Hippen, K. L., Curtsinger, J., Defor, T., Levine, B. L., June, C. H., Rubinstein, P., McGlave, P. B., Blazar, B. R. & Wagner, J. E. (2011) Infusion of ex vivo expanded T regulatory cells in adults transplanted with umbilical cord blood: safety profile and detection kinetics. *Blood*, 117, 1061-70.
- Bullens, D. M., Truyen, E., Coteur, L., Dilissen, E., Hellings, P. W., Dupont, L. J. & Ceuppens, J. L. (2006) IL-17 mRNA in sputum of asthmatic patients: linking T cell driven inflammation and granulocytic influx? *Respir Res*, 7, 135.
- Burns, J., Bartholomew, B. & Lobo, S. (1999) Isolation of myelin basic protein-specific T cells predominantly from the memory T-cell compartment in multiple sclerosis. *Ann Neurol*, 45, 33-9.

- Burrows, B., Martinez, F. D., Halonen, M., Barbee, R. A. & Cline, M. G. (1989) Association of asthma with serum IgE levels and skin-test reactivity to allergens. *N Engl J Med*, 320, 271-7.
- Burtrum, D. B., Kim, S., Dudley, E. C., Hayday, A. C. & Petrie, H. T. (1996) TCR gene recombination and alpha beta-gamma delta lineage divergence: productive TCR-beta rearrangement is neither exclusive nor preclusive of gamma delta cell development. *J Immunol*, 157, 4293-6.
- Busse, W. W. & Lemanske, R. F., Jr. (2001) Asthma. *N Engl J Med*, 344, 350-62.
- Campbell, D. J. & Koch, M. A. (2011) Phenotypical and functional specialization of FOXP3+ regulatory T cells. *Nat Rev Immunol*, 11, 119-30.
- Cao, X., Cai, S. F., Fehniger, T. A., Song, J., Collins, L. I., Piwnica-Worms, D. R. & Ley, T. J. (2007) Granzyme B and perforin are important for regulatory T cell-mediated suppression of tumor clearance. *Immunity*, 27, 635-46.
- Cao, X., Leonard, K., Collins, L. I., Cai, S. F., Mayer, J. C., Payton, J. E., Walter, M. J., Piwnica-Worms, D., Schreiber, R. D. & Ley, T. J. (2009) Interleukin 12 stimulates IFN-gamma-mediated inhibition of tumor-induced regulatory T-cell proliferation and enhances tumor clearance. *Cancer Res*, 69, 8700-9.
- Cardoso, W. V., Williams, M. C., Mitsialis, S. A., Joyce-Brady, M., Rishi, A. K. & Brody, J. S. (1995) Retinoic acid induces changes in the pattern of airway branching and alters epithelial cell differentiation in the developing lung in vitro. *Am J Respir Cell Mol Biol*, 12, 464-76.
- Carrier, Y., Yuan, J., Kuchroo, V. K. & Weiner, H. L. (2007) Th3 cells in peripheral tolerance. I. Induction of Foxp3-positive regulatory T cells by Th3 cells derived from TGF-beta T cell-transgenic mice. *J Immunol*, 178, 179-85.
- Carroll, M. C. (2004) The complement system in regulation of adaptive immunity. *Nat Immunol*, 5, 981-6.
- Casares, N., Arribillaga, L., Sarobe, P., Dotor, J., Lopez-Diaz de Cerio, A., Melero, I., Prieto, J., Borrás-Cuesta, F. & Lasarte, J. J. (2003) CD4+/CD25+ regulatory cells inhibit activation of tumor-primed CD4+ T cells with IFN-gamma-dependent antiangiogenic activity, as well as long-lasting tumor immunity elicited by peptide vaccination. *J Immunol*, 171, 5931-9.
- Cederbom, L., Hall, H. & Ivars, F. (2000) CD4+CD25+ regulatory T cells down-regulate co-stimulatory molecules on antigen-presenting cells. *Eur J Immunol*, 30, 1538-43.
- Challoner, P. B., Smith, K. T., Parker, J. D., MacLeod, D. L., Coulter, S. N., Rose, T. M., Schultz, E. R., Bennett, J. L., Garber, R. L., Chang, M. & et al. (1995) Plaque-associated expression of human herpesvirus 6 in multiple sclerosis. *Proc Natl Acad Sci U S A*, 92, 7440-4.
- Chang, Y. J., Kim, H. Y., Albacker, L. A., Baumgarth, N., McKenzie, A. N., Smith, D. E., Dekruyff, R. H. & Umetsu, D. T. (2011) Innate lymphoid cells mediate influenza-induced airway hyper-reactivity independently of adaptive immunity. *Nat Immunol*, 12, 631-8.
- Charil, A., Zijdenbos, A. P., Taylor, J., Boelman, C., Worsley, K. J., Evans, A. C. & Dagher, A. (2003) Statistical mapping analysis of lesion location and neurological disability in multiple sclerosis: application to 452 patient data sets. *Neuroimage*, 19, 532-44.

- Chaturvedi, V., Collison, L. W., Guy, C. S., Workman, C. J. & Vignali, D. A. (2011) Cutting edge: Human regulatory T cells require IL-35 to mediate suppression and infectious tolerance. *J Immunol*, 186, 6661-6.
- Chaudhry, A., Rudra, D., Treuting, P., Samstein, R. M., Liang, Y., Kas, A. & Rudensky, A. Y. (2009) CD4⁺ regulatory T cells control TH17 responses in a Stat3-dependent manner. *Science*, 326, 986-91.
- Chen, L. C., Delgado, J. C., Jensen, P. E. & Chen, X. (2009) Direct expansion of human allospecific FoxP3⁺CD4⁺ regulatory T cells with allogeneic B cells for therapeutic application. *J Immunol*, 183, 4094-102.
- Chen, Q., Kim, Y. C., Laurence, A., Punkosdy, G. A. & Shevach, E. M. (2011) IL-2 Controls the Stability of Foxp3 Expression in TGF- β -Induced Foxp3⁺ T Cells In Vivo. *J Immunol*, 186, 6329-37.
- Chen, W., Jin, W., Hardegen, N., Lei, K. J., Li, L., Marinos, N., McGrady, G. & Wahl, S. M. (2003) Conversion of peripheral CD4⁺CD25⁻ naive T cells to CD4⁺CD25⁺ regulatory T cells by TGF- β induction of transcription factor Foxp3. *J Exp Med*, 198, 1875-86.
- Christ, M., McCartney-Francis, N. L., Kulkarni, A. B., Ward, J. M., Mizel, D. E., Mackall, C. L., Gress, R. E., Hines, K. L., Tian, H., Karlsson, S. & et al. (1994) Immune dysregulation in TGF- β 1-deficient mice. *J Immunol*, 153, 1936-46.
- Ciofani, M. & Zuniga-Pflucker, J. C. (2010) Determining gammadelta versus alphass T cell development. *Nat Rev Immunol*, 10, 657-63.
- Clark, L. B., Appleby, M. W., Brunkow, M. E., Wilkinson, J. E., Ziegler, S. F. & Ramsdell, F. (1999) Cellular and molecular characterization of the scurfy mouse mutant. *J Immunol*, 162, 2546-54.
- Clarke, S. L., Betts, G. J., Plant, A., Wright, K. L., El-Shanawany, T. M., Harrop, R., Torkington, J., Rees, B. I., Williams, G. T., Gallimore, A. M. & Godkin, A. J. (2006) CD4⁺CD25⁺FOXP3⁺ regulatory T cells suppress anti-tumor immune responses in patients with colorectal cancer. *PLoS One*, 1, e129.
- ClinicalTrials.gov (2012) T1DM Immunotherapy Using CD4⁺CD127^{lo/-}CD25⁺ Polyclonal Tregs.
- Cobbold, S. P., Castejon, R., Adams, E., Zelenika, D., Graca, L., Humm, S. & Waldmann, H. (2004) Induction of foxP3⁺ regulatory T cells in the periphery of T cell receptor transgenic mice tolerized to transplants. *J Immunol*, 172, 6003-10.
- Codarri, L., Gyulveszi, G., Tosevski, V., Hesske, L., Fontana, A., Magnenat, L., Suter, T. & Becher, B. (2011) RORgammat drives production of the cytokine GM-CSF in helper T cells, which is essential for the effector phase of autoimmune neuroinflammation. *Nat Immunol*, 12, 560-7.
- Cohn, L., Homer, R. J., Marinov, A., Rankin, J. & Bottomly, K. (1997) Induction of airway mucus production By T helper 2 (Th2) cells: a critical role for interleukin 4 in cell recruitment but not mucus production. *J Exp Med*, 186, 1737-47.
- Collison, L. W., Workman, C. J., Kuo, T. T., Boyd, K., Wang, Y., Vignali, K. M., Cross, R., Sehy, D., Blumberg, R. S. & Vignali, D. A. (2007) The inhibitory cytokine IL-35 contributes to regulatory T-cell function. *Nature*, 450, 566-9.

- Conrad, M. L., Yildirim, A. O., Sonar, S. S., Kilic, A., Sudowe, S., Lunow, M., Teich, R., Renz, H. & Garn, H. (2009) Comparison of adjuvant and adjuvant-free murine experimental asthma models. *Clin Exp Allergy*, 39, 1246-54.
- Consortium, A. a. N. Z. M. S. G. (2009) Genome-wide association study identifies new multiple sclerosis susceptibility loci on chromosomes 12 and 20. *Nat Genet*, 41, 824-8.
- Correale, J., Rush, C., Amengual, A. & Goicochea, M. T. (2005) Mitoxantrone as rescue therapy in worsening relapsing-remitting MS patients receiving IFN-beta. *J Neuroimmunol*, 162, 173-83.
- Correale, J. & Villa, A. (2008) Isolation and characterization of CD8+ regulatory T cells in multiple sclerosis. *J Neuroimmunol*, 195, 121-34.
- Coyle, A. J., Le Gros, G., Bertrand, C., Tsuyuki, S., Heusser, C. H., Kopf, M. & Anderson, G. P. (1995) Interleukin-4 is required for the induction of lung Th2 mucosal immunity. *Am J Respir Cell Mol Biol*, 13, 54-9.
- Crotty, S. (2011) Follicular helper CD4 T cells (TFH). *Annu Rev Immunol*, 29, 621-63.
- Cua, D. J., Hutchins, B., LaFace, D. M., Stohlman, S. A. & Coffman, R. L. (2001) Central nervous system expression of IL-10 inhibits autoimmune encephalomyelitis. *J Immunol*, 166, 602-8.
- Cua, D. J., Sherlock, J., Chen, Y., Murphy, C. A., Joyce, B., Seymour, B., Lucian, L., To, W., Kwan, S., Churakova, T., Zurawski, S., Wiekowski, M., Lira, S. A., Gorman, D., Kastelein, R. A. & Sedgwick, J. D. (2003) Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature*, 421, 744-8.
- Curotto de Lafaille, M. A. & Lafaille, J. J. (2009) Natural and adaptive foxp3+ regulatory T cells: more of the same or a division of labor? *Immunity*, 30, 626-35.
- D'Ambrosio, D., Mariani, M., Panina-Bordignon, P. & Sinigaglia, F. (2001) Chemokines and their receptors guiding T lymphocyte recruitment in lung inflammation. *Am J Respir Crit Care Med*, 164, 1266-75.
- Dardalhon, V., Awasthi, A., Kwon, H., Galileos, G., Gao, W., Sobel, R. A., Mitsdoerffer, M., Strom, T. B., Elyaman, W., Ho, I. C., Khoury, S., Oukka, M. & Kuchroo, V. K. (2008) IL-4 inhibits TGF-beta-induced Foxp3+ T cells and, together with TGF-beta, generates IL-9+ IL-10+ Foxp3(-) effector T cells. *Nat Immunol*, 9, 1347-55.
- Davidson, A. & Diamond, B. (2001) Autoimmune diseases. *N Engl J Med*, 345, 340-50.
- Davidson, T. S. & Shevach, E. M. (2011) Polyclonal Treg cells modulate T effector cell trafficking. *Eur J Immunol*, 41, 2862-70.
- Davis, M. M. & Bjorkman, P. J. (1988) T-cell antigen receptor genes and T-cell recognition. *Nature*, 334, 395-402.
- de Jong, J. M., Schuurhuis, D. H., Ioan-Facsinay, A., Welling, M. M., Camps, M. G., van der Voort, E. I., Huizinga, T. W., Ossendorp, F., Verbeek, J. S. & Toes, R. E. (2006) Dendritic cells, but not macrophages or B cells, activate major histocompatibility complex class II-restricted CD4+ T cells upon immune-complex uptake in vivo. *Immunology*, 119, 499-506.

- de la Rosa, M., Rutz, S., Dorninger, H. & Scheffold, A. (2004) Interleukin-2 is essential for CD4⁺CD25⁺ regulatory T cell function. *Eur J Immunol*, 34, 2480-8.
- Deaglio, S., Dwyer, K. M., Gao, W., Friedman, D., Usheva, A., Erat, A., Chen, J. F., Enjyoji, K., Linden, J., Oukka, M., Kuchroo, V. K., Strom, T. B. & Robson, S. C. (2007) Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *J Exp Med*, 204, 1257-65.
- Dejean, A. S., Beisner, D. R., Ch'en, I. L., Kerdiles, Y. M., Babour, A., Arden, K. C., Castrillon, D. H., DePinho, R. A. & Hedrick, S. M. (2009) Transcription factor Foxo3 controls the magnitude of T cell immune responses by modulating the function of dendritic cells. *Nat Immunol*, 10, 504-13.
- Deknuydt, F., Bioley, G., Valmori, D. & Ayyoub, M. (2009) IL-1 β and IL-2 convert human Treg into T(H)17 cells. *Clin Immunol*, 131, 298-307.
- Del Prete, G. F., De Carli, M., D'Elia, M. M., Maestrelli, P., Ricci, M., Fabbri, L. & Romagnani, S. (1993) Allergen exposure induces the activation of allergen-specific Th2 cells in the airway mucosa of patients with allergic respiratory disorders. *Eur J Immunol*, 23, 1445-9.
- Delarasse, C., Daubas, P., Mars, L. T., Vizler, C., Litzenburger, T., Iglesias, A., Bauer, J., Della Gaspera, B., Schubart, A., Decker, L., Dimitri, D., Roussel, G., Dierich, A., Amor, S., Dautigny, A., Liblau, R. & Pham-Dinh, D. (2003) Myelin/oligodendrocyte glycoprotein-deficient (MOG-deficient) mice reveal lack of immune tolerance to MOG in wild-type mice. *J Clin Invest*, 112, 544-53.
- Derbinski, J., Schulte, A., Kyewski, B. & Klein, L. (2001) Promiscuous gene expression in medullary thymic epithelial cells mirrors the peripheral self. *Nat Immunol*, 2, 1032-9.
- Di Ianni, M., Falzetti, F., Carotti, A., Terenzi, A., Castellino, F., Bonifacio, E., Del Papa, B., Zei, T., Ostini, R. I., Cecchini, D., Aloisi, T., Perruccio, K., Ruggeri, L., Balucani, C., Pierini, A., Sportoletti, P., Aristei, C., Falini, B., Reisner, Y., Velardi, A., Aversa, F. & Martelli, M. F. (2011) Tregs prevent GVHD and promote immune reconstitution in HLA-haploidentical transplantation. *Blood*, 117, 3921-8.
- Doherty, T. A., Soroosh, P., Broide, D. H. & Croft, M. (2009) CD4⁺ cells are required for chronic eosinophilic lung inflammation but not airway remodeling. *Am J Physiol Lung Cell Mol Physiol*, 296, L229-35.
- Douek, D. C., Corley, K. T., Zal, T., Mellor, A., Dyson, P. J. & Altmann, D. M. (1996) Negative selection by endogenous antigen and superantigen occurs at multiple thymic sites. *Int Immunol*, 8, 1413-20.
- Drulovic, J., Mostarica-Stojkovic, M., Levic, Z., Stojavljevic, N., Pravica, V. & Mesaros, S. (1997) Interleukin-12 and tumor necrosis factor- α levels in cerebrospinal fluid of multiple sclerosis patients. *J Neurol Sci*, 147, 145-50.
- Duda, P. W., Schmied, M. C., Cook, S. L., Krieger, J. I. & Hafler, D. A. (2000) Glatiramer acetate (Copaxone) induces degenerate, Th2-polarized immune responses in patients with multiple sclerosis. *J Clin Invest*, 105, 967-76.
- Duhen, T., Duhen, R., Lanzavecchia, A., Sallusto, F. & Campbell, D. J. (2012) Functionally distinct subsets of human FOXP3⁺ Treg cells that phenotypically mirror effector Th cells. *Blood*, 119, 4430-40.

- Durelli, L., Conti, L., Clerico, M., Boselli, D., Contessa, G., Ripellino, P., Ferrero, B., Eid, P. & Novelli, F. (2009) T-helper 17 cells expand in multiple sclerosis and are inhibited by interferon-beta. *Ann Neurol*, 65, 499-509.
- Dyment, D. A., Ebers, G. C. & Sadovnick, A. D. (2004) Genetics of multiple sclerosis. *Lancet Neurol*, 3, 104-10.
- Edinger, M. & Hoffmann, P. (2011) Regulatory T cells in stem cell transplantation: strategies and first clinical experiences. *Curr Opin Immunol*, 23, 679-84.
- El-Behi, M., Ciric, B., Dai, H., Yan, Y., Cullimore, M., Safavi, F., Zhang, G. X., Dittel, B. N. & Rostami, A. (2011) The encephalitogenicity of T(H)17 cells is dependent on IL-1- and IL-23-induced production of the cytokine GM-CSF. *Nat Immunol*, 12, 568-75.
- Elhofy, A., Depaolo, R. W., Lira, S. A., Lukacs, N. W. & Karpus, W. J. (2009) Mice deficient for CCR6 fail to control chronic experimental autoimmune encephalomyelitis. *J Neuroimmunol*, 213, 91-9.
- Elyaman, W., Bradshaw, E. M., Uyttenhove, C., Dardalhon, V., Awasthi, A., Imitola, J., Bettelli, E., Oukka, M., van Snick, J., Renauld, J. C., Kuchroo, V. K. & Khoury, S. J. (2009) IL-9 induces differentiation of TH17 cells and enhances function of FoxP3+ natural regulatory T cells. *Proc Natl Acad Sci U S A*, 106, 12885-90.
- Engelhardt, B. & Kappos, L. (2008) Natalizumab: targeting alpha4-integrins in multiple sclerosis. *Neurodegener Dis*, 5, 16-22.
- Ermann, J. & Fathman, C. G. (2003) Costimulatory signals controlling regulatory T cells. *Proc Natl Acad Sci U S A*, 100, 15292-3.
- Esiri, M. M. (1977) Immunoglobulin-containing cells in multiple-sclerosis plaques. *Lancet*, 2, 478.
- Eyerich, S., Eyerich, K., Pennino, D., Carbone, T., Nasorri, F., Pallotta, S., Cianfarani, F., Odorisio, T., Traidl-Hoffmann, C., Behrendt, H., Durham, S. R., Schmidt-Weber, C. B. & Cavani, A. (2009) Th22 cells represent a distinct human T cell subset involved in epidermal immunity and remodeling. *J Clin Invest*, 119, 3573-85.
- Fahy, J. V., Fleming, H. E., Wong, H. H., Liu, J. T., Su, J. Q., Reimann, J., Fick, R. B., Jr. & Boushey, H. A. (1997) The effect of an anti-IgE monoclonal antibody on the early- and late-phase responses to allergen inhalation in asthmatic subjects. *Am J Respir Crit Care Med*, 155, 1828-34.
- Fallarino, F., Grohmann, U., Hwang, K. W., Orabona, C., Vacca, C., Bianchi, R., Belladonna, M. L., Fioretti, M. C., Alegre, M. L. & Puccetti, P. (2003) Modulation of tryptophan catabolism by regulatory T cells. *Nat Immunol*, 4, 1206-12.
- Fanta, C. H. (2009) Asthma. *N Engl J Med*, 360, 1002-14.
- Ferber, I. A., Brocke, S., Taylor-Edwards, C., Ridgway, W., Dinisco, C., Steinman, L., Dalton, D. & Fathman, C. G. (1996) Mice with a disrupted IFN-gamma gene are susceptible to the induction of experimental autoimmune encephalomyelitis (EAE). *J Immunol*, 156, 5-7.
- Fidler, J. M., DeJoy, S. Q. & Gibbons, J. J., Jr. (1986) Selective immunomodulation by the antineoplastic agent mitoxantrone. I. Suppression of B lymphocyte function. *J Immunol*, 137, 727-32.

- Fillatreau, S., Sweeney, C. H., McGeachy, M. J., Gray, D. & Anderton, S. M. (2002) B cells regulate autoimmunity by provision of IL-10. *Nat Immunol*, 3, 944-50.
- Firan, M., Dhillon, S., Estess, P. & Siegelman, M. H. (2006) Suppressor activity and potency among regulatory T cells is discriminated by functionally active CD44. *Blood*, 107, 619-27.
- Floess, S., Freyer, J., Siewert, C., Baron, U., Olek, S., Polansky, J., Schlawe, K., Chang, H. D., Bopp, T., Schmitt, E., Klein-Hessling, S., Serfling, E., Hamann, A. & Huehn, J. (2007) Epigenetic control of the foxp3 locus in regulatory T cells. *PLoS Biol*, 5, e38.
- Fontenot, J. D., Gavin, M. A. & Rudensky, A. Y. (2003) Foxp3 programs the development and function of CD4⁺CD25⁺ regulatory T cells. *Nat Immunol*, 4, 330-6.
- Fontenot, J. D., Rasmussen, J. P., Gavin, M. A. & Rudensky, A. Y. (2005a) A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat Immunol*, 6, 1142-51.
- Fontenot, J. D., Rasmussen, J. P., Williams, L. M., Dooley, J. L., Farr, A. G. & Rudensky, A. Y. (2005b) Regulatory T cell lineage specification by the forkhead transcription factor foxp3. *Immunity*, 22, 329-41.
- Foster, P. S., Hogan, S. P., Ramsay, A. J., Matthaei, K. I. & Young, I. G. (1996) Interleukin 5 deficiency abolishes eosinophilia, airways hyperreactivity, and lung damage in a mouse asthma model. *J Exp Med*, 183, 195-201.
- Foster, P. S., Yang, M., Herbert, C. & Kumar, R. K. (2002) CD4⁺ T-lymphocytes regulate airway remodeling and hyper-reactivity in a mouse model of chronic asthma. *Lab Invest*, 82, 455-62.
- Fox, E. J. (2006) Management of worsening multiple sclerosis with mitoxantrone: a review. *Clin Ther*, 28, 461-74.
- Franklin, R. J. & Ffrench-Constant, C. (2008) Remyelination in the CNS: from biology to therapy. *Nat Rev Neurosci*, 9, 839-55.
- Freeman, G. J., Gray, G. S., Gimmi, C. D., Lombard, D. B., Zhou, L. J., White, M., Fingerhuth, J. D., Gribben, J. G. & Nadler, L. M. (1991) Structure, expression, and T cell costimulatory activity of the murine homologue of the human B lymphocyte activation antigen B7. *J Exp Med*, 174, 625-31.
- Freeman, G. J., Long, A. J., Iwai, Y., Bourque, K., Chernova, T., Nishimura, H., Fitz, L. J., Malenkovich, N., Okazaki, T., Byrne, M. C., Horton, H. F., Fouser, L., Carter, L., Ling, V., Bowman, M. R., Carreno, B. M., Collins, M., Wood, C. R. & Honjo, T. (2000) Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *J Exp Med*, 192, 1027-34.
- Friese, M. A. & Fugger, L. (2005) Autoreactive CD8⁺ T cells in multiple sclerosis: a new target for therapy? *Brain*, 128, 1747-63.
- Fritzsche, B., Haas, J., Konig, F., Kunz, P., Fritzsche, E., Poschl, J., Krammer, P. H., Bruck, W., Suri-Payer, E. & Wildemann, B. (2011) Intracerebral human regulatory T cells: analysis of CD4⁺ CD25⁺ FOXP3⁺ T cells in brain lesions and cerebrospinal fluid of multiple sclerosis patients. *PLoS One*, 6, e17988.
- Furtado, G. C., Marcondes, M. C., Latkowski, J. A., Tsai, J., Wensky, A. & Lafaille, J. J. (2008) Swift entry of myelin-specific T lymphocytes into the central

- nervous system in spontaneous autoimmune encephalomyelitis. *J Immunol*, 181, 4648-55.
- Gadina, M. & O'Shea, J. J. (2009) Immune modulation: Turncoat regulatory T cells. *Nat Med*, 15, 1365.
- Games & Cooperative, T. M. S. G. (2003) A meta-analysis of whole genome linkage screens in multiple sclerosis. *J Neuroimmunol*, 143, 39-46.
- Gavett, S. H., Chen, X., Finkelman, F. & Wills-Karp, M. (1994) Depletion of murine CD4+ T lymphocytes prevents antigen-induced airway hyperreactivity and pulmonary eosinophilia. *Am J Respir Cell Mol Biol*, 10, 587-93.
- Genain, C. P., Cannella, B., Hauser, S. L. & Raine, C. S. (1999) Identification of autoantibodies associated with myelin damage in multiple sclerosis. *Nat Med*, 5, 170-5.
- Gerblich, A. A., Salik, H. & Schuyler, M. R. (1991) Dynamic T-cell changes in peripheral blood and bronchoalveolar lavage after antigen bronchoprovocation in asthmatics. *Am Rev Respir Dis*, 143, 533-7.
- Gershon, R. K. & Kondo, K. (1970) Cell interactions in the induction of tolerance: the role of thymic lymphocytes. *Immunology*, 18, 723-37.
- Gershon, R. K. & Kondo, K. (1971) Infectious immunological tolerance. *Immunology*, 21, 903-14.
- Godfrey, V. L., Wilkinson, J. E. & Russell, L. B. (1991) X-linked lymphoreticular disease in the scurfy (sf) mutant mouse. *Am J Pathol*, 138, 1379-87.
- Gondek, D. C., Lu, L. F., Quezada, S. A., Sakaguchi, S. & Noelle, R. J. (2005) Cutting edge: contact-mediated suppression by CD4+CD25+ regulatory cells involves a granzyme B-dependent, perforin-independent mechanism. *J Immunol*, 174, 1783-6.
- Gonzalo, J. A., Lloyd, C. M., Kremer, L., Finger, E., Martinez, A. C., Siegelman, M. H., Cybulsky, M. & Gutierrez-Ramos, J. C. (1996) Eosinophil recruitment to the lung in a murine model of allergic inflammation. The role of T cells, chemokines, and adhesion receptors. *J Clin Invest*, 98, 2332-45.
- Gottschalk, R. A., Corse, E. & Allison, J. P. (2012) Expression of Helios in peripherally induced Foxp3+ regulatory T cells. *J Immunol*, 188, 976-80.
- Goverman, J. (2009) Autoimmune T cell responses in the central nervous system. *Nat Rev Immunol*, 9, 393-407.
- Goverman, J., Woods, A., Larson, L., Weiner, L. P., Hood, L. & Zaller, D. M. (1993) Transgenic mice that express a myelin basic protein-specific T cell receptor develop spontaneous autoimmunity. *Cell*, 72, 551-60.
- Grakoui, A., Bromley, S. K., Sumen, C., Davis, M. M., Shaw, A. S., Allen, P. M. & Dustin, M. L. (1999) The immunological synapse: a molecular machine controlling T cell activation. *Science*, 285, 221-7.
- Greer, J. M., Kuchroo, V. K., Sobel, R. A. & Lees, M. B. (1992) Identification and characterization of a second encephalitogenic determinant of myelin proteolipid protein (residues 178-191) for SJL mice. *J Immunol*, 149, 783-8.
- Greter, M., Heppner, F. L., Lemos, M. P., Odermatt, B. M., Goebels, N., Laufer, T., Noelle, R. J. & Becher, B. (2005) Dendritic cells permit immune invasion of the CNS in an animal model of multiple sclerosis. *Nat Med*, 11, 328-34.
- Grohmann, U., Orabona, C., Fallarino, F., Vacca, C., Calcinaro, F., Falorni, A., Candeloro, P., Belladonna, M. L., Bianchi, R., Fioretti, M. C. & Puccetti, P.

- (2002) CTLA-4-Ig regulates tryptophan catabolism in vivo. *Nat Immunol*, 3, 1097-101.
- Gross, J. A., Callas, E. & Allison, J. P. (1992) Identification and distribution of the costimulatory receptor CD28 in the mouse. *J Immunol*, 149, 380-8.
- Groux, H. (2003) Type 1 T-regulatory cells: their role in the control of immune responses. *Transplantation*, 75, 8S-12S.
- Groux, H., O'Garra, A., Bigler, M., Rouleau, M., Antonenko, S., de Vries, J. E. & Roncarolo, M. G. (1997) A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature*, 389, 737-42.
- Gueders, M. M., Paulissen, G., Crahay, C., Quesada-Calvo, F., Hacha, J., Van Hove, C., Tournoy, K., Louis, R., Foidart, J. M., Noel, A. & Cataldo, D. D. (2009) Mouse models of asthma: a comparison between C57BL/6 and BALB/c strains regarding bronchial responsiveness, inflammation, and cytokine production. *Inflamm Res*, 58, 845-54.
- Guermonprez, P., Valladeau, J., Zitvogel, L., Thery, C. & Amigorena, S. (2002) Antigen presentation and T cell stimulation by dendritic cells. *Annu Rev Immunol*, 20, 621-67.
- Gundel, R. H., Letts, L. G. & Gleich, G. J. (1991) Human eosinophil major basic protein induces airway constriction and airway hyperresponsiveness in primates. *J Clin Invest*, 87, 1470-3.
- Gutcher, I. & Becher, B. (2007) APC-derived cytokines and T cell polarization in autoimmune inflammation. *J Clin Invest*, 117, 1119-27.
- Haas, J., Hug, A., Viehover, A., Fritzsching, B., Falk, C. S., Filser, A., Vetter, T., Milkova, L., Korporal, M., Fritz, B., Storch-Hagenlocher, B., Krammer, P. H., Suri-Payer, E. & Wildemann, B. (2005) Reduced suppressive effect of CD4+CD25high regulatory T cells on the T cell immune response against myelin oligodendrocyte glycoprotein in patients with multiple sclerosis. *Eur J Immunol*, 35, 3343-52.
- Hafler, D. A., Compston, A., Sawcer, S., Lander, E. S., Daly, M. J., De Jager, P. L., de Bakker, P. I., Gabriel, S. B., Mirel, D. B., Ivinson, A. J., Pericak-Vance, M. A., Gregory, S. G., Rioux, J. D., McCauley, J. L., Haines, J. L., Barcellos, L. F., Cree, B., Oksenberg, J. R. & Hauser, S. L. (2007) Risk alleles for multiple sclerosis identified by a genomewide study. *N Engl J Med*, 357, 851-62.
- Hammad, H., Chieppa, M., Perros, F., Willart, M. A., Germain, R. N. & Lambrecht, B. N. (2009) House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells. *Nat Med*, 15, 410-6.
- Hammad, H., Plantinga, M., Deswarte, K., Pouliot, P., Willart, M. A., Kool, M., Muskens, F. & Lambrecht, B. N. (2010) Inflammatory dendritic cells--not basophils--are necessary and sufficient for induction of Th2 immunity to inhaled house dust mite allergen. *J Exp Med*, 207, 2097-111.
- Hansen, G., Berry, G., DeKruyff, R. H. & Umetsu, D. T. (1999) Allergen-specific Th1 cells fail to counterbalance Th2 cell-induced airway hyperreactivity but cause severe airway inflammation. *J Clin Invest*, 103, 175-83.
- Hartl, D., Koller, B., Mehlhorn, A. T., Reinhardt, D., Nicolai, T., Schendel, D. J., Griese, M. & Krauss-Etschmann, S. (2007) Quantitative and functional impairment of pulmonary CD4+CD25hi regulatory T cells in pediatric asthma. *J Allergy Clin Immunol*, 119, 1258-66.

- Hartung, H. P., Gonsette, R., Konig, N., Kwiecinski, H., Guseo, A., Morrissey, S. P., Krapf, H. & Zwingers, T. (2002) Mitoxantrone in progressive multiple sclerosis: a placebo-controlled, double-blind, randomised, multicentre trial. *Lancet*, 360, 2018-25.
- Hauser, S. L., Waubant, E., Arnold, D. L., Vollmer, T., Antel, J., Fox, R. J., Bar-Or, A., Panzara, M., Sarkar, N., Agarwal, S., Langer-Gould, A. & Smith, C. H. (2008) B-cell depletion with rituximab in relapsing-remitting multiple sclerosis. *N Engl J Med*, 358, 676-88.
- Hayes, C. E. (2000) Vitamin D: a natural inhibitor of multiple sclerosis. *Proc Nutr Soc*, 59, 531-5.
- Hegazy, A. N., Peine, M., Helmstetter, C., Panse, I., Frohlich, A., Bergthaler, A., Flatz, L., Pinschewer, D. D., Radbruch, A. & Lohning, M. (2010) Interferons direct Th2 cell reprogramming to generate a stable GATA-3(+)T-bet(+) cell subset with combined Th2 and Th1 cell functions. *Immunity*, 32, 116-28.
- Hemmer, B., Archelos, J. J. & Hartung, H. P. (2002) New concepts in the immunopathogenesis of multiple sclerosis. *Nat Rev Neurosci*, 3, 291-301.
- Hippen, K. L., Merkel, S. C., Schirm, D. K., Sieben, C. M., Sumstad, D., Kadidlo, D. M., McKenna, D. H., Bromberg, J. S., Levine, B. L., Riley, J. L., June, C. H., Scheinberg, P., Douek, D. C., Miller, J. S., Wagner, J. E. & Blazar, B. R. (2011) Massive ex vivo expansion of human natural regulatory T cells (T(regs)) with minimal loss of in vivo functional activity. *Sci Transl Med*, 3, 83ra41.
- Hoffmann, P., Boeld, T. J., Eder, R., Huehn, J., Floess, S., Wieczorek, G., Olek, S., Dietmaier, W., Andreesen, R. & Edinger, M. (2009) Loss of FOXP3 expression in natural human CD4⁺CD25⁺ regulatory T cells upon repetitive in vitro stimulation. *Eur J Immunol*, 39, 1088-97.
- Hoffmann, P., Eder, R., Kunz-Schughart, L. A., Andreesen, R. & Edinger, M. (2004) Large-scale in vitro expansion of polyclonal human CD4⁺CD25^{high} regulatory T cells. *Blood*, 104, 895-903.
- Hogan, S. P., Koskinen, A. & Foster, P. S. (1997) Interleukin-5 and eosinophils induce airway damage and bronchial hyperreactivity during allergic airway inflammation in BALB/c mice. *Immunol Cell Biol*, 75, 284-8.
- Hogan, S. P., Koskinen, A., Matthaei, K. I., Young, I. G. & Foster, P. S. (1998) Interleukin-5-producing CD4⁺ T cells play a pivotal role in aeroallergen-induced eosinophilia, bronchial hyperreactivity, and lung damage in mice. *Am J Respir Crit Care Med*, 157, 210-8.
- Holgate, S. T., Arshad, H. S., Roberts, G. C., Howarth, P. H., Thurner, P. & Davies, D. E. (2009) A new look at the pathogenesis of asthma. *Clin Sci (Lond)*, 118, 439-50.
- Holloway, J. W., Yang, I. A. & Holgate, S. T. (2010) Genetics of allergic disease. *J Allergy Clin Immunol*, 125, S81-94.
- Holmoy, T., Kvale, E. O. & Vartdal, F. (2004) Cerebrospinal fluid CD4⁺ T cells from a multiple sclerosis patient cross-recognize Epstein-Barr virus and myelin basic protein. *J Neurovirol*, 10, 278-83.
- Holt, P. G., Schon-Hegrad, M. A., Oliver, J., Holt, B. J. & McMenamin, P. G. (1990) A contiguous network of dendritic antigen-presenting cells within the respiratory epithelium. *Int Arch Allergy Appl Immunol*, 91, 155-9.

- Honma, K., Kohno, Y., Saito, K., Shimojo, N., Horiuchi, T., Hayashi, H., Suzuki, N., Hosoya, T., Tsunoo, H. & Niimi, H. (1996) Allergenic epitopes of ovalbumin (OVA) in patients with hen's egg allergy: inhibition of basophil histamine release by haptenic ovalbumin peptide. *Clin Exp Immunol*, 103, 446-53.
- Hori, S., Nomura, T. & Sakaguchi, S. (2003) Control of regulatory T cell development by the transcription factor Foxp3. *Science*, 299, 1057-61.
- Hsieh, C. S., Macatonia, S. E., Tripp, C. S., Wolf, S. F., O'Garra, A. & Murphy, K. M. (1993) Development of TH1 CD4+ T cells through IL-12 produced by *Listeria*-induced macrophages. *Science*, 260, 547-9.
- Hsieh, C. S., Zheng, Y., Liang, Y., Fontenot, J. D. & Rudensky, A. Y. (2006) An intersection between the self-reactive regulatory and nonregulatory T cell receptor repertoires. *Nat Immunol*, 7, 401-10.
- Huehn, J., Polansky, J. K. & Hamann, A. (2009) Epigenetic control of FOXP3 expression: the key to a stable regulatory T-cell lineage? *Nat Rev Immunol*, 9, 83-9.
- Huehn, J., Siegmund, K., Lehmann, J. C., Siewert, C., Haubold, U., Feuerer, M., Debes, G. F., Lauber, J., Frey, O., Przybylski, G. K., Niesner, U., de la Rosa, M., Schmidt, C. A., Brauer, R., Buer, J., Scheffold, A. & Hamann, A. (2004) Developmental stage, phenotype, and migration distinguish naive- and effector/memory-like CD4+ regulatory T cells. *J Exp Med*, 199, 303-13.
- Huseby, E. S., Liggitt, D., Brabb, T., Schnabel, B., Ohlen, C. & Goverman, J. (2001) A pathogenic role for myelin-specific CD8(+) T cells in a model for multiple sclerosis. *J Exp Med*, 194, 669-76.
- Hutchinson, M. (2007) Natalizumab: A new treatment for relapsing remitting multiple sclerosis. *Ther Clin Risk Manag*, 3, 259-68.
- Huter, E. N., Stummvoll, G. H., DiPaolo, R. J., Glass, D. D. & Shevach, E. M. (2008) Cutting edge: antigen-specific TGF beta-induced regulatory T cells suppress Th17-mediated autoimmune disease. *J Immunol*, 181, 8209-13.
- Iglesias, A., Bauer, J., Litzenburger, T., Schubart, A. & Linington, C. (2001) T- and B-cell responses to myelin oligodendrocyte glycoprotein in experimental autoimmune encephalomyelitis and multiple sclerosis. *Glia*, 36, 220-34.
- Illes, Z., Kondo, T., Yokoyama, K., Ohashi, T., Tabira, T. & Yamamura, T. (1999) Identification of autoimmune T cells among in vivo expanded CD25+ T cells in multiple sclerosis. *J Immunol*, 162, 1811-7.
- Ingulli, E., Ulman, D. R., Lucido, M. M. & Jenkins, M. K. (2002) In situ analysis reveals physical interactions between CD11b+ dendritic cells and antigen-specific CD4 T cells after subcutaneous injection of antigen. *J Immunol*, 169, 2247-52.
- Itoh, M., Takahashi, T., Sakaguchi, N., Kuniyasu, Y., Shimizu, J., Otsuka, F. & Sakaguchi, S. (1999) Thymus and autoimmunity: production of CD25+CD4+ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance. *J Immunol*, 162, 5317-26.
- Ivanov, II, McKenzie, B. S., Zhou, L., Tadokoro, C. E., Lepelley, A., Lafaille, J. J., Cua, D. J. & Littman, D. R. (2006) The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell*, 126, 1121-33.
- Jacobs, L. D., Cookfair, D. L., Rudick, R. A., Herndon, R. M., Richert, J. R., Salazar, A. M., Fischer, J. S., Goodkin, D. E., Granger, C. V., Simon, J. H., Alam, J.

- J., Bartoszak, D. M., Bourdette, D. N., Braiman, J., Brownschidle, C. M., Coats, M. E., Cohan, S. L., Dougherty, D. S., Kinkel, R. P., Mass, M. K., Munschauer, F. E., 3rd, Priore, R. L., Pullicino, P. M., Scherokman, B. J., Whitham, R. H. & et al. (1996) Intramuscular interferon beta-1a for disease progression in relapsing multiple sclerosis. The Multiple Sclerosis Collaborative Research Group (MSCRG). *Ann Neurol*, 39, 285-94.
- Jaffar, Z., Sivakuru, T. & Roberts, K. (2004) CD4+CD25+ T cells regulate airway eosinophilic inflammation by modulating the Th2 cell phenotype. *J Immunol*, 172, 3842-9.
- Jameson, S. C., Hogquist, K. A. & Bevan, M. J. (1995) Positive selection of thymocytes. *Annu Rev Immunol*, 13, 93-126.
- Jenkins, M. K., Pardoll, D. M., Mizuguchi, J., Chused, T. M. & Schwartz, R. H. (1987) Molecular events in the induction of a nonresponsive state in interleukin 2-producing helper T-lymphocyte clones. *Proc Natl Acad Sci U S A*, 84, 5409-13.
- John, M., Lim, S., Seybold, J., Jose, P., Robichaud, A., O'Connor, B., Barnes, P. J. & Chung, K. F. (1998) Inhaled corticosteroids increase interleukin-10 but reduce macrophage inflammatory protein-1alpha, granulocyte-macrophage colony-stimulating factor, and interferon-gamma release from alveolar macrophages in asthma. *Am J Respir Crit Care Med*, 157, 256-62.
- Jonuleit, H., Schmitt, E., Schuler, G., Knop, J. & Enk, A. H. (2000) Induction of interleukin 10-producing, nonproliferating CD4(+) T cells with regulatory properties by repetitive stimulation with allogeneic immature human dendritic cells. *J Exp Med*, 192, 1213-22.
- Jordan, M. S., Boesteanu, A., Reed, A. J., Petrone, A. L., Hohenbeck, A. E., Lerman, M. A., Naji, A. & Caton, A. J. (2001) Thymic selection of CD4+CD25+ regulatory T cells induced by an agonist self-peptide. *Nat Immunol*, 2, 301-6.
- Kaiko, G. E., Horvat, J. C., Beagley, K. W. & Hansbro, P. M. (2008) Immunological decision-making: how does the immune system decide to mount a helper T-cell response? *Immunology*, 123, 326-38.
- Kamanaka, M., Huber, S., Zenewicz, L. A., Gagliani, N., Rathinam, C., O'Connor, W., Jr., Wan, Y. Y., Nakae, S., Iwakura, Y., Hao, L. & Flavell, R. A. (2011) Memory/effector (CD45RB(lo)) CD4 T cells are controlled directly by IL-10 and cause IL-22-dependent intestinal pathology. *J Exp Med*, 208, 1027-40.
- Kang, S. G., Piniacki, R. J., Hogenesch, H., Lim, H. W., Wiebke, E., Braun, S. E., Matsumoto, S. & Kim, C. H. (2007) Identification of a chemokine network that recruits FoxP3(+) regulatory T cells into chronically inflamed intestine. *Gastroenterology*, 132, 966-81.
- Kaplan, M. H., Sun, Y. L., Hoey, T. & Grusby, M. J. (1996) Impaired IL-12 responses and enhanced development of Th2 cells in Stat4-deficient mice. *Nature*, 382, 174-7.
- Kappos, L. (2002) Interferon beta-1a and beta-1b for treatment of multiple sclerosis. *Lancet*, 360, 1428; author reply 1428-9.
- Kapsenberg, M. L. (2003) Dendritic-cell control of pathogen-driven T-cell polarization. *Nat Rev Immunol*, 3, 984-93.
- Kashiwada, M., Levy, D. M., McKeag, L., Murray, K., Schroder, A. J., Canfield, S. M., Traver, G. & Rothman, P. B. (2010) IL-4-induced transcription factor

- NFIL3/E4BP4 controls IgE class switching. *Proc Natl Acad Sci U S A*, 107, 821-6.
- Kearley, J., Barker, J. E., Robinson, D. S. & Lloyd, C. M. (2005) Resolution of airway inflammation and hyperreactivity after in vivo transfer of CD4+CD25+ regulatory T cells is interleukin 10 dependent. *J Exp Med*, 202, 1539-47.
- Kebir, H., Ifergan, I., Alvarez, J. I., Bernard, M., Poirier, J., Arbour, N., Duquette, P. & Prat, A. (2009) Preferential recruitment of interferon-gamma-expressing TH17 cells in multiple sclerosis. *Ann Neurol*, 66, 390-402.
- Kerfoot, S. M., Long, E. M., Hickey, M. J., Andonegui, G., Lapointe, B. M., Zanardo, R. C., Bonder, C., James, W. G., Robbins, S. M. & Kubes, P. (2004) TLR4 contributes to disease-inducing mechanisms resulting in central nervous system autoimmune disease. *J Immunol*, 173, 7070-7.
- Kim, B. S., Kim, I. K., Park, Y. J., Kim, Y. S., Kim, Y. J., Chang, W. S., Lee, Y. S., Kweon, M. N., Chung, Y. & Kang, C. Y. (2010) Conversion of Th2 memory cells into Foxp3+ regulatory T cells suppressing Th2-mediated allergic asthma. *Proc Natl Acad Sci U S A*, 107, 8742-7.
- Kim, J. M., Rasmussen, J. P. & Rudensky, A. Y. (2007) Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice. *Nat Immunol*, 8, 191-7.
- Kincses, Z. T., Ropele, S., Jenkinson, M., Khalil, M., Petrovic, K., Loitfelder, M., Langkammer, C., Aspeck, E., Wallner-Blazek, M., Fuchs, S., Jehna, M., Schmidt, R., Vecsei, L., Fazekas, F. & Enzinger, C. (2010) Lesion probability mapping to explain clinical deficits and cognitive performance in multiple sclerosis. *Mult Scler*, 17, 681-9.
- Kisielow, P., Teh, H. S., Bluthmann, H. & von Boehmer, H. (1988) Positive selection of antigen-specific T cells in thymus by restricting MHC molecules. *Nature*, 335, 730-3.
- Klion, A. D. & Nutman, T. B. (2004) The role of eosinophils in host defense against helminth parasites. *J Allergy Clin Immunol*, 113, 30-7.
- Knoechel, B., Lohr, J., Kahn, E., Bluestone, J. A. & Abbas, A. K. (2005) Sequential development of interleukin 2-dependent effector and regulatory T cells in response to endogenous systemic antigen. *J Exp Med*, 202, 1375-86.
- Kobie, J. J., Shah, P. R., Yang, L., Rebhahn, J. A., Fowell, D. J. & Mosmann, T. R. (2006) T regulatory and primed uncommitted CD4 T cells express CD73, which suppresses effector CD4 T cells by converting 5'-adenosine monophosphate to adenosine. *J Immunol*, 177, 6780-6.
- Koch, M. A., Tucker-Heard, G., Perdue, N. R., Killebrew, J. R., Urdahl, K. B. & Campbell, D. J. (2009) The transcription factor T-bet controls regulatory T cell homeostasis and function during type 1 inflammation. *Nat Immunol*, 10, 595-602.
- Kohm, A. P., Carpentier, P. A., Anger, H. A. & Miller, S. D. (2002) Cutting edge: CD4+CD25+ regulatory T cells suppress antigen-specific autoreactive immune responses and central nervous system inflammation during active experimental autoimmune encephalomyelitis. *J Immunol*, 169, 4712-6.
- Kolls, J. K. (2010) Th17 cells in mucosal immunity and tissue inflammation. *Semin Immunopathol*, 32, 1-2.

- Komatsu, N., Mariotti-Ferrandiz, M. E., Wang, Y., Malissen, B., Waldmann, H. & Hori, S. (2009) Heterogeneity of natural Foxp3⁺ T cells: a committed regulatory T-cell lineage and an uncommitted minor population retaining plasticity. *Proc Natl Acad Sci U S A*, 106, 1903-8.
- Komiyama, Y., Nakae, S., Matsuki, T., Nambu, A., Ishigame, H., Kakuta, S., Sudo, K. & Iwakura, Y. (2006) IL-17 plays an important role in the development of experimental autoimmune encephalomyelitis. *J Immunol*, 177, 566-73.
- Korn, T., Reddy, J., Gao, W., Bettelli, E., Awasthi, A., Petersen, T. R., Backstrom, B. T., Sobel, R. A., Wucherpfennig, K. W., Strom, T. B., Oukka, M. & Kuchroo, V. K. (2007) Myelin-specific regulatory T cells accumulate in the CNS but fail to control autoimmune inflammation. *Nat Med*, 13, 423-31.
- Kretschmer, K., Apostolou, I., Hawiger, D., Khazaie, K., Nussenzweig, M. C. & von Boehmer, H. (2005) Inducing and expanding regulatory T cell populations by foreign antigen. *Nat Immunol*, 6, 1219-27.
- Krummel, M. F. & Allison, J. P. (1995) Pillars article: CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. The journal of experimental medicine. 1995. 182: 459-465. *J Immunol*, 187, 3459-65.
- Kuhns, M. S., Davis, M. M. & Garcia, K. C. (2006) Deconstructing the form and function of the TCR/CD3 complex. *Immunity*, 24, 133-9.
- Kullberg, M. C., Hay, V., Cheever, A. W., Mamura, M., Sher, A., Letterio, J. J., Shevach, E. M. & Piccirillo, C. A. (2005) TGF-beta1 production by CD4⁺ CD25⁺ regulatory T cells is not essential for suppression of intestinal inflammation. *Eur J Immunol*, 35, 2886-95.
- Kurtzke, J. F. (1991) Multiple sclerosis: changing times. *Neuroepidemiology*, 10, 1-8.
- Lahl, K., Loddenkemper, C., Drouin, C., Freyer, J., Arnason, J., Eberl, G., Hamann, A., Wagner, H., Huehn, J. & Sparwasser, T. (2007) Selective depletion of Foxp3⁺ regulatory T cells induces a scurfy-like disease. *J Exp Med*, 204, 57-63.
- Lambrecht, B. N. & Hammad, H. (2009) Biology of lung dendritic cells at the origin of asthma. *Immunity*, 31, 412-24.
- Langer-Gould, A., Atlas, S. W., Green, A. J., Bollen, A. W. & Pelletier, D. (2005) Progressive multifocal leukoencephalopathy in a patient treated with natalizumab. *N Engl J Med*, 353, 375-81.
- Langrish, C. L., Chen, Y., Blumenschein, W. M., Mattson, J., Basham, B., Sedgwick, J. D., McClanahan, T., Kastelein, R. A. & Cua, D. J. (2005) IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J Exp Med*, 201, 233-40.
- Lassmann, H. & Ransohoff, R. M. (2004) The CD4-Th1 model for multiple sclerosis: a critical [correction of crucial] re-appraisal. *Trends Immunol*, 25, 132-7.
- Le Bras, S. & Geha, R. S. (2006) IPEX and the role of Foxp3 in the development and function of human Tregs. *J Clin Invest*, 116, 1473-5.
- Leckie, M. J., ten Brinke, A., Khan, J., Diamant, Z., O'Connor, B. J., Walls, C. M., Mathur, A. K., Cowley, H. C., Chung, K. F., Djukanovic, R., Hansel, T. T., Holgate, S. T., Sterk, P. J. & Barnes, P. J. (2000) Effects of an interleukin-5 blocking monoclonal antibody on eosinophils, airway hyper-responsiveness, and the late asthmatic response. *Lancet*, 356, 2144-8.

- Lee, Y. H., Ishida, Y., Rifa'i, M., Shi, Z., Isobe, K. & Suzuki, H. (2008) Essential role of CD8+CD122+ regulatory T cells in the recovery from experimental autoimmune encephalomyelitis. *J Immunol*, 180, 825-32.
- Lee, Y. K., Turner, H., Maynard, C. L., Oliver, J. R., Chen, D., Elson, C. O. & Weaver, C. T. (2009) Late developmental plasticity in the T helper 17 lineage. *Immunity*, 30, 92-107.
- Leech, M. D., Benson, R. A., De Vries, A., Fitch, P. M. & Howie, S. E. (2007) Resolution of Der p1-induced allergic airway inflammation is dependent on CD4+CD25+Foxp3+ regulatory cells. *J Immunol*, 179, 7050-8.
- Leech, M. D., Carrillo-Vico, A., Liblau, R. S. & Anderton, S. M. (2011) Recognition of a high affinity MHC class I-restricted epitope of myelin oligodendrocyte glycoprotein by CD8(+) T cells derived from autoantigen-deficient mice. *Front Immunol*, 2, 17.
- Lehmann, P. V., Sercarz, E. E., Forsthuber, T., Dayan, C. M. & Gammon, G. (1993) Determinant spreading and the dynamics of the autoimmune T-cell repertoire. *Immunol Today*, 14, 203-8.
- Leonard, J. P., Waldburger, K. E. & Goldman, S. J. (1996) Regulation of experimental autoimmune encephalomyelitis by interleukin-12. *Ann N Y Acad Sci*, 795, 216-26.
- Levings, M. K., Sangregorio, R., Sartirana, C., Moschin, A. L., Battaglia, M., Orban, P. C. & Roncarolo, M. G. (2002) Human CD25+CD4+ T suppressor cell clones produce transforming growth factor beta, but not interleukin 10, and are distinct from type 1 T regulatory cells. *J Exp Med*, 196, 1335-46.
- Lewkowich, I. P., Herman, N. S., Schleifer, K. W., Dance, M. P., Chen, B. L., Dienger, K. M., Sproles, A. A., Shah, J. S., Kohl, J., Belkaid, Y. & Wills-Karp, M. (2005) CD4+CD25+ T cells protect against experimentally induced asthma and alter pulmonary dendritic cell phenotype and function. *J Exp Med*, 202, 1549-61.
- Liang, B., Workman, C., Lee, J., Chew, C., Dale, B. M., Colonna, L., Flores, M., Li, N., Schweighoffer, E., Greenberg, S., Tybulewicz, V., Vignali, D. & Clynes, R. (2008) Regulatory T cells inhibit dendritic cells by lymphocyte activation gene-3 engagement of MHC class II. *J Immunol*, 180, 5916-26.
- Lighvani, A. A., Frucht, D. M., Jankovic, D., Yamane, H., Aliberti, J., Hissong, B. D., Nguyen, B. V., Gadina, M., Sher, A., Paul, W. E. & O'Shea, J. J. (2001) T-bet is rapidly induced by interferon-gamma in lymphoid and myeloid cells. *Proc Natl Acad Sci U S A*, 98, 15137-42.
- Linden, J. (2006) New insights into the regulation of inflammation by adenosine. *J Clin Invest*, 116, 1835-7.
- Linsley, P. S., Greene, J. L., Tan, P., Bradshaw, J., Ledbetter, J. A., Anasetti, C. & Damle, N. K. (1992) Coexpression and functional cooperation of CTLA-4 and CD28 on activated T lymphocytes. *J Exp Med*, 176, 1595-604.
- Linthicum, D. S., Munoz, J. J. & Blaskett, A. (1982) Acute experimental autoimmune encephalomyelitis in mice. I. Adjuvant action of Bordetella pertussis is due to vasoactive amine sensitization and increased vascular permeability of the central nervous system. *Cell Immunol*, 73, 299-310.
- Lio, C. W. & Hsieh, C. S. (2008) A two-step process for thymic regulatory T cell development. *Immunity*, 28, 100-11.

- Liston, A., Lesage, S., Wilson, J., Peltonen, L. & Goodnow, C. C. (2003) Aire regulates negative selection of organ-specific T cells. *Nat Immunol*, 4, 350-4.
- Liu, G. Y., Fairchild, P. J., Smith, R. M., Prowle, J. R., Kioussis, D. & Wraith, D. C. (1995) Low avidity recognition of self-antigen by T cells permits escape from central tolerance. *Immunity*, 3, 407-15.
- Liu, W., Putnam, A. L., Xu-Yu, Z., Szot, G. L., Lee, M. R., Zhu, S., Gottlieb, P. A., Kapranov, P., Gingeras, T. R., Fazekas de St Groth, B., Clayberger, C., Soper, D. M., Ziegler, S. F. & Bluestone, J. A. (2006) CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. *J Exp Med*, 203, 1701-11.
- Liu, Y., Zhang, P., Li, J., Kulkarni, A. B., Perruche, S. & Chen, W. (2008) A critical function for TGF-beta signaling in the development of natural CD4+CD25+Foxp3+ regulatory T cells. *Nat Immunol*, 9, 632-40.
- Livak, F., Tourigny, M., Schatz, D. G. & Petrie, H. T. (1999) Characterization of TCR gene rearrangements during adult murine T cell development. *J Immunol*, 162, 2575-80.
- Lohoff, M., Mittrucker, H. W., Prechtel, S., Bischof, S., Sommer, F., Kock, S., Ferrick, D. A., Duncan, G. S., Gessner, A. & Mak, T. W. (2002) Dysregulated T helper cell differentiation in the absence of interferon regulatory factor 4. *Proc Natl Acad Sci U S A*, 99, 11808-12.
- Longley, R. L., Woods, A., Fleetwood, A., Cowling, G. J., Gallagher, J. T. & Couchman, J. R. (1999) Control of morphology, cytoskeleton and migration by syndecan-4. *J Cell Sci*, 112 (Pt 20), 3421-31.
- Lu, L., Zhou, X., Wang, J., Zheng, S. G. & Horwitz, D. A. (2010) Characterization of protective human CD4CD25 FOXP3 regulatory T cells generated with IL-2, TGF-beta and retinoic acid. *PLoS One*, 5, e15150.
- Lu, Y., Malmhall, C., Sjostrand, M., Radinger, M., O'Neil, S. E., Lotvall, J. & Bossios, A. (2011) Expansion of CD4CD25 and CD25 T-Bet, GATA-3, Foxp3 and RORgammat Cells in Allergic Inflammation, Local Lung Distribution and Chemokine Gene Expression. *PLoS One*, 6, e19889.
- Lubberts, E. (2008) IL-17/Th17 targeting: on the road to prevent chronic destructive arthritis? *Cytokine*, 41, 84-91.
- Luhder, F., Hoglund, P., Allison, J. P., Benoist, C. & Mathis, D. (1998) Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) regulates the unfolding of autoimmune diabetes. *J Exp Med*, 187, 427-32.
- Mackenzie, K. J. (2011) Peptide Immunotherapy in Models of Allergic Airways Disease. Thesis (PhD). University of Edinburgh.
- Malhotra, N., Robertson, E. & Kang, J. (2010) SMAD2 is essential for TGF beta-mediated Th17 cell generation. *J Biol Chem*, 285, 29044-8.
- Mangan, P. R., Harrington, L. E., O'Quinn, D. B., Helms, W. S., Bullard, D. C., Elson, C. O., Hatton, R. D., Wahl, S. M., Schoeb, T. R. & Weaver, C. T. (2006) Transforming growth factor-beta induces development of the T(H)17 lineage. *Nature*, 441, 231-4.
- Mantel, P. Y., Kuipers, H., Boyman, O., Rhyner, C., Ouaked, N., Ruckert, B., Karagiannidis, C., Lambrecht, B. N., Hendriks, R. W., Cramer, R., Akdis, C. A., Blaser, K. & Schmidt-Weber, C. B. (2007) GATA3-driven Th2 responses inhibit TGF-beta1-induced FOXP3 expression and the formation of regulatory T cells. *PLoS Biol*, 5, e329.

- Markowitz, C. E. (2007) Interferon-beta: mechanism of action and dosing issues. *Neurology*, 68, S8-11.
- Marrack, P., McKee, A. S. & Munks, M. W. (2009) Towards an understanding of the adjuvant action of aluminium. *Nat Rev Immunol*, 9, 287-93.
- Marson, A., Kretschmer, K., Frampton, G. M., Jacobsen, E. S., Polansky, J. K., MacIsaac, K. D., Levine, S. S., Fraenkel, E., von Boehmer, H. & Young, R. A. (2007) Foxp3 occupancy and regulation of key target genes during T-cell stimulation. *Nature*, 445, 931-5.
- Mattner, F., Smioldo, S., Galbiati, F., Muller, M., Di Lucia, P., Poliani, P. L., Martino, G., Panina-Bordignon, P. & Adorini, L. (2000) Inhibition of Th1 development and treatment of chronic-relapsing experimental allergic encephalomyelitis by a non-hypercalcemic analogue of 1,25-dihydroxyvitamin D(3). *Eur J Immunol*, 30, 498-508.
- Mauri, C., Gray, D., Mushtaq, N. & Londei, M. (2003) Prevention of arthritis by interleukin 10-producing B cells. *J Exp Med*, 197, 489-501.
- McFarland, H. F. & Martin, R. (2007) Multiple sclerosis: a complicated picture of autoimmunity. *Nat Immunol*, 8, 913-9.
- McGeachy, M. J., Stephens, L. A. & Anderton, S. M. (2005) Natural recovery and protection from autoimmune encephalomyelitis: contribution of CD4+CD25+ regulatory cells within the central nervous system. *J Immunol*, 175, 3025-32.
- McGee, H. S. & Agrawal, D. K. (2009) Naturally occurring and inducible T-regulatory cells modulating immune response in allergic asthma. *Am J Respir Crit Care Med*, 180, 211-25.
- McHugh, R. S., Whitters, M. J., Piccirillo, C. A., Young, D. A., Shevach, E. M., Collins, M. & Byrne, M. C. (2002) CD4(+)CD25(+) immunoregulatory T cells: gene expression analysis reveals a functional role for the glucocorticoid-induced TNF receptor. *Immunity*, 16, 311-23.
- McPherson, R. C. (2012) The effect of tolerogenic peptide administration on pathogenic antigen-experienced T cells. Thesis (PhD). University of Edinburgh.
- MedicalNewsToday (2011) Antigen-Specific Treg Cells For Crohn's Disease Treatment - Trial.
- Medoff, B. D., Thomas, S. Y. & Luster, A. D. (2008) T cell trafficking in allergic asthma: the ins and outs. *Annu Rev Immunol*, 26, 205-32.
- Mendel, I., Kerlero de Rosbo, N. & Ben-Nun, A. (1995) A myelin oligodendrocyte glycoprotein peptide induces typical chronic experimental autoimmune encephalomyelitis in H-2b mice: fine specificity and T cell receptor V beta expression of encephalitogenic T cells. *Eur J Immunol*, 25, 1951-9.
- Metcalf, D. D., Baram, D. & Mekori, Y. A. (1997) Mast cells. *Physiol Rev*, 77, 1033-79.
- Miller, G., Pillarisetty, V. G., Shah, A. B., Lahrs, S., Xing, Z. & DeMatteo, R. P. (2002) Endogenous granulocyte-macrophage colony-stimulating factor overexpression in vivo results in the long-term recruitment of a distinct dendritic cell population with enhanced immunostimulatory function. *J Immunol*, 169, 2875-85.
- Miller, M. J., Safrina, O., Parker, I. & Cahalan, M. D. (2004) Imaging the single cell dynamics of CD4+ T cell activation by dendritic cells in lymph nodes. *J Exp Med*, 200, 847-56.

- Minagar, A. & Alexander, J. S. (2003) Blood-brain barrier disruption in multiple sclerosis. *Mult Scler*, 9, 540-9.
- Minagar, A., Alexander, J. S., Sahraian, M. A. & Zivadinov, R. (2010) Alemtuzumab and multiple sclerosis: therapeutic application. *Expert Opin Biol Ther*, 10, 421-9.
- Misra, N., Bayry, J., Lacroix-Desmazes, S., Kazatchkine, M. D. & Kaveri, S. V. (2004) Cutting edge: human CD4+CD25+ T cells restrain the maturation and antigen-presenting function of dendritic cells. *J Immunol*, 172, 4676-80.
- Miyara, M., Yoshioka, Y., Kitoh, A., Shima, T., Wing, K., Niwa, A., Parizot, C., Taflin, C., Heike, T., Valeyre, D., Mathian, A., Nakahata, T., Yamaguchi, T., Nomura, T., Ono, M., Amoura, Z., Gorochoy, G. & Sakaguchi, S. (2009) Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor. *Immunity*, 30, 899-911.
- Modigliani, Y., Coutinho, A., Pereira, P., Le Douarin, N., Thomas-Vaslin, V., Burlen-Defranoux, O., Salaun, J. & Bandeira, A. (1996) Establishment of tissue-specific tolerance is driven by regulatory T cells selected by thymic epithelium. *Eur J Immunol*, 26, 1807-15.
- Mosmann, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. A. & Coffman, R. L. (1986) Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol*, 136, 5-14.
- Mozo, L., Suarez, A. & Gutierrez, C. (2004) Glucocorticoids up-regulate constitutive interleukin-10 production by human monocytes. *Clin Exp Allergy*, 34, 406-12.
- Mueller, D. L. (2010) Mechanisms maintaining peripheral tolerance. *Nat Immunol*, 11, 21-7.
- Mueller, D. L., Jenkins, M. K. & Schwartz, R. H. (1989) An accessory cell-derived costimulatory signal acts independently of protein kinase C activation to allow T cell proliferation and prevent the induction of unresponsiveness. *J Immunol*, 142, 2617-28.
- Muller, M., Carter, S. L., Hofer, M. J., Manders, P., Getts, D. R., Getts, M. T., Dreykluft, A., Lu, B., Gerard, C., King, N. J. & Campbell, I. L. (2007) CXCR3 signaling reduces the severity of experimental autoimmune encephalomyelitis by controlling the parenchymal distribution of effector and regulatory T cells in the central nervous system. *J Immunol*, 179, 2774-86.
- Munger, K. L., Zhang, S. M., O'Reilly, E., Hernan, M. A., Olek, M. J., Willett, W. C. & Ascherio, A. (2004) Vitamin D intake and incidence of multiple sclerosis. *Neurology*, 62, 60-5.
- Murphy, K. M., Heimberger, A. B. & Loh, D. Y. (1990) Induction by antigen of intrathymic apoptosis of CD4+CD8+TCRlo thymocytes in vivo. *Science*, 250, 1720-3.
- Myers, L., Croft, M., Kwon, B. S., Mittler, R. S. & Vella, A. T. (2005) Peptide-specific CD8 T regulatory cells use IFN-gamma to elaborate TGF-beta-based suppression. *J Immunol*, 174, 7625-32.
- Nagamine, K., Peterson, P., Scott, H. S., Kudoh, J., Minoshima, S., Heino, M., Krohn, K. J., Lalioti, M. D., Mullis, P. E., Antonarakis, S. E., Kawasaki, K., Asakawa, S., Ito, F. & Shimizu, N. (1997) Positional cloning of the APECED gene. *Nat Genet*, 17, 393-8.

- Nakamura, K., Kitani, A. & Strober, W. (2001) Cell contact-dependent immunosuppression by CD4(+)CD25(+) regulatory T cells is mediated by cell surface-bound transforming growth factor beta. *J Exp Med*, 194, 629-44.
- Nakayama, T. & Yamashita, M. (2010) The TCR-mediated signaling pathways that control the direction of helper T cell differentiation. *Semin Immunol*, 22, 303-9.
- Nathan, C. (2006) Neutrophils and immunity: challenges and opportunities. *Nat Rev Immunol*, 6, 173-82.
- Neill, D. R., Wong, S. H., Bellosi, A., Flynn, R. J., Daly, M., Langford, T. K., Bucks, C., Kane, C. M., Fallon, P. G., Pannell, R., Jolin, H. E. & McKenzie, A. N. (2010) Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature*, 464, 1367-70.
- Neufert, C., Becker, C., Wirtz, S., Fantini, M. C., Weigmann, B., Galle, P. R. & Neurath, M. F. (2007) IL-27 controls the development of inducible regulatory T cells and Th17 cells via differential effects on STAT1. *Eur J Immunol*, 37, 1809-16.
- Nguyen, T. L., Sullivan, N. L., Ebel, M., Teague, R. M. & Dipaolo, R. J. (2011) Antigen-Specific TGF- β -Induced Regulatory T Cells Secrete Chemokines, Regulate T Cell Trafficking, and Suppress Ongoing Autoimmunity. *J Immunol*.
- Nials, A. T. & Uddin, S. (2008) Mouse models of allergic asthma: acute and chronic allergen challenge. *Dis Model Mech*, 1, 213-20.
- Nicolae, D., Cox, N. J., Lester, L. A., Schneider, D., Tan, Z., Billstrand, C., Kuldanek, S., Donfack, J., Kogut, P., Patel, N. M., Goodenbour, J., Howard, T., Wolf, R., Koppelman, G. H., White, S. R., Parry, R., Postma, D. S., Meyers, D., Bleeker, E. R., Hunt, J. S., Solway, J. & Ober, C. (2005) Fine mapping and positional candidate studies identify HLA-G as an asthma susceptibility gene on chromosome 6p21. *Am J Hum Genet*, 76, 349-57.
- Nicoletti, F., Patti, F., Cocuzza, C., Zacccone, P., Nicoletti, A., Di Marco, R. & Reggio, A. (1996) Elevated serum levels of interleukin-12 in chronic progressive multiple sclerosis. *J Neuroimmunol*, 70, 87-90.
- Niedbala, W., Wei, X. Q., Cai, B., Hueber, A. J., Leung, B. P., McInnes, I. B. & Liew, F. Y. (2007) IL-35 is a novel cytokine with therapeutic effects against collagen-induced arthritis through the expansion of regulatory T cells and suppression of Th17 cells. *Eur J Immunol*, 37, 3021-9.
- Nocentini, G. & Riccardi, C. (2009) GITR: a modulator of immune response and inflammation. *Adv Exp Med Biol*, 647, 156-73.
- Noseworthy, J. H., Lucchinetti, C., Rodriguez, M. & Weinshenker, B. G. (2000) Multiple sclerosis. *N Engl J Med*, 343, 938-52.
- Nurieva, R. I., Chung, Y., Martinez, G. J., Yang, X. O., Tanaka, S., Matskevitch, T. D., Wang, Y. H. & Dong, C. (2009) Bcl6 mediates the development of T follicular helper cells. *Science*, 325, 1001-5.
- O'Connor, R. A. & Anderton, S. M. (2008) Foxp3+ regulatory T cells in the control of experimental CNS autoimmune disease. *J Neuroimmunol*, 193, 1-11.
- O'Connor, R. A., Floess, S., Huehn, J., Jones, S. A. & Anderton, S. M. (2012) Foxp3(+) Treg cells in the inflamed CNS are insensitive to IL-6-driven IL-17 production. *Eur J Immunol*, 42, 1174-9.

- O'Connor, R. A., Leech, M. D., Suffner, J., Hammerling, G. J. & Anderton, S. M. (2010) Myelin-reactive, TGF-beta-induced regulatory T cells can be programmed to develop Th1-like effector function but remain less proinflammatory than myelin-reactive Th1 effectors and can suppress pathogenic T cell clonal expansion in vivo. *J Immunol*, 185, 7235-43.
- O'Connor, R. A., Malpass, K. H. & Anderton, S. M. (2007) The inflamed central nervous system drives the activation and rapid proliferation of Foxp3+ regulatory T cells. *J Immunol*, 179, 958-66.
- O'Connor, R. A., Prendergast, C. T., Sabatos, C. A., Lau, C. W., Leech, M. D., Wraith, D. C. & Anderton, S. M. (2008) Cutting edge: Th1 cells facilitate the entry of Th17 cells to the central nervous system during experimental autoimmune encephalomyelitis. *J Immunol*, 181, 3750-4.
- O'Donnell, M. C., Ackerman, S. J., Gleich, G. J. & Thomas, L. L. (1983) Activation of basophil and mast cell histamine release by eosinophil granule major basic protein. *J Exp Med*, 157, 1981-91.
- O'Garra, A., Barrat, F. J., Castro, A. G., Vicari, A. & Hawrylowicz, C. (2008) Strategies for use of IL-10 or its antagonists in human disease. *Immunol Rev*, 223, 114-31.
- O'Shea, J. J. & Paul, W. E. (2010) Mechanisms underlying lineage commitment and plasticity of helper CD4+ T cells. *Science*, 327, 1098-102.
- Oderup, C., Cederbom, L., Makowska, A., Cilio, C. M. & Ivars, F. (2006) Cytotoxic T lymphocyte antigen-4-dependent down-modulation of costimulatory molecules on dendritic cells in CD4+ CD25+ regulatory T-cell-mediated suppression. *Immunology*, 118, 240-9.
- Ohnishi, T., Sur, S., Collins, D. S., Fish, J. E., Gleich, G. J. & Peters, S. P. (1993) Eosinophil survival activity identified as interleukin-5 is associated with eosinophil recruitment and degranulation and lung injury twenty-four hours after segmental antigen lung challenge. *J Allergy Clin Immunol*, 92, 607-15.
- Oldenhove, G., Bouladoux, N., Wohlfert, E. A., Hall, J. A., Chou, D., Dos Santos, L., O'Brien, S., Blank, R., Lamb, E., Natarajan, S., Kastenmayer, R., Hunter, C., Grigg, M. E. & Belkaid, Y. (2009) Decrease of Foxp3+ Treg cell number and acquisition of effector cell phenotype during lethal infection. *Immunity*, 31, 772-86.
- Oldstone, M. B. (1987) Molecular mimicry and autoimmune disease. *Cell*, 50, 819-20.
- Olerup, O. & Hillert, J. (1991) HLA class II-associated genetic susceptibility in multiple sclerosis: a critical evaluation. *Tissue Antigens*, 38, 1-15.
- Olivares-Villagomez, D., Wensky, A. K., Wang, Y. & Lafaille, J. J. (2000) Repertoire requirements of CD4+ T cells that prevent spontaneous autoimmune encephalomyelitis. *J Immunol*, 164, 5499-507.
- Onishi, Y., Fehervari, Z., Yamaguchi, T. & Sakaguchi, S. (2008) Foxp3+ natural regulatory T cells preferentially form aggregates on dendritic cells in vitro and actively inhibit their maturation. *Proc Natl Acad Sci U S A*, 105, 10113-8.
- Oppmann, B., Lesley, R., Blom, B., Timans, J. C., Xu, Y., Hunte, B., Vega, F., Yu, N., Wang, J., Singh, K., Zonin, F., Vaisberg, E., Churakova, T., Liu, M., Gorman, D., Wagner, J., Zurawski, S., Liu, Y., Abrams, J. S., Moore, K. W., Rennick, D., de Waal-Malefyt, R., Hannum, C., Bazan, J. F. & Kastelein, R. A. (2000) Novel p19 protein engages IL-12p40 to form a cytokine, IL-23,

- with biological activities similar as well as distinct from IL-12. *Immunity*, 13, 715-25.
- Oriss, T. B., McCarthy, S. A., Morel, B. F., Campana, M. A. & Morel, P. A. (1997) Crossregulation between T helper cell (Th)1 and Th2: inhibition of Th2 proliferation by IFN-gamma involves interference with IL-1. *J Immunol*, 158, 3666-72.
- Ostroukhova, M., Seguin-Devaux, C., Oriss, T. B., Dixon-McCarthy, B., Yang, L., Ameredes, B. T., Corcoran, T. E. & Ray, A. (2004) Tolerance induced by inhaled antigen involves CD4(+) T cells expressing membrane-bound TGF-beta and FOXP3. *J Clin Invest*, 114, 28-38.
- Ozenci, V., Kouwenhoven, M., Huang, Y. M., Kivisakk, P. & Link, H. (2000) Multiple sclerosis is associated with an imbalance between tumour necrosis factor-alpha (TNF-alpha)- and IL-10-secreting blood cells that is corrected by interferon-beta (IFN-beta) treatment. *Clin Exp Immunol*, 120, 147-53.
- Pacholczyk, R. & Kern, J. (2008) The T-cell receptor repertoire of regulatory T cells. *Immunology*, 125, 450-8.
- Pacholczyk, R., Kern, J., Singh, N., Iwashima, M., Kraj, P. & Ignatowicz, L. (2007) Nonself-antigens are the cognate specificities of Foxp3+ regulatory T cells. *Immunity*, 27, 493-504.
- Pandiyani, P., Zheng, L., Ishihara, S., Reed, J. & Lenardo, M. J. (2007) CD4+CD25+Foxp3+ regulatory T cells induce cytokine deprivation-mediated apoptosis of effector CD4+ T cells. *Nat Immunol*, 8, 1353-62.
- Panitch, H. S., Hirsch, R. L., Haley, A. S. & Johnson, K. P. (1987) Exacerbations of multiple sclerosis in patients treated with gamma interferon. *Lancet*, 1, 893-5.
- Panzer, M., Sitte, S., Wirth, S., Drexler, I., Sparwasser, T. & Voehringer, D. (2012) Rapid in vivo conversion of effector T cells into Th2 cells during helminth infection. *J Immunol*, 188, 615-23.
- Parker, D. C. (1993) T cell-dependent B cell activation. *Annu Rev Immunol*, 11, 331-60.
- Peng, G., Guo, Z., Kiniwa, Y., Voo, K. S., Peng, W., Fu, T., Wang, D. Y., Li, Y., Wang, H. Y. & Wang, R. F. (2005) Toll-like receptor 8-mediated reversal of CD4+ regulatory T cell function. *Science*, 309, 1380-4.
- Perry, L. L., Barzaga-Gilbert, E. & Trotter, J. L. (1991) T cell sensitization to proteolipid protein in myelin basic protein-induced relapsing experimental allergic encephalomyelitis. *J Neuroimmunol*, 33, 7-15.
- Petrie, H. T. & Zuniga-Pflucker, J. C. (2007) Zoned out: functional mapping of stromal signaling microenvironments in the thymus. *Annu Rev Immunol*, 25, 649-79.
- Piccio, L., Rossi, B., Scarpini, E., Laudanna, C., Giagulli, C., Issekutz, A. C., Vestweber, D., Butcher, E. C. & Constantin, G. (2002) Molecular mechanisms involved in lymphocyte recruitment in inflamed brain microvessels: critical roles for P-selectin glycoprotein ligand-1 and heterotrimeric G(i)-linked receptors. *J Immunol*, 168, 1940-9.
- Piccirillo, C. A., Letterio, J. J., Thornton, A. M., McHugh, R. S., Mamura, M., Mizuhara, H. & Shevach, E. M. (2002) CD4(+)CD25(+) regulatory T cells can mediate suppressor function in the absence of transforming growth factor beta1 production and responsiveness. *J Exp Med*, 196, 237-46.

- Pillai, M. R., Collison, L. W., Wang, X., Finkelstein, D., Rehg, J. E., Boyd, K., Szymczak-Workman, A. L., Doggett, T., Griffith, T. S., Ferguson, T. A. & Vignali, D. A. (2011) The plasticity of regulatory T cell function. *J Immunol*, 187, 4987-97.
- Polman, C. H., O'Connor, P. W., Havrdova, E., Hutchinson, M., Kappos, L., Miller, D. H., Phillips, J. T., Lublin, F. D., Giovannoni, G., Wajgt, A., Toal, M., Lynn, F., Panzara, M. A. & Sandrock, A. W. (2006) A randomized, placebo-controlled trial of natalizumab for relapsing multiple sclerosis. *N Engl J Med*, 354, 899-910.
- Ponomarev, E. D., Shriver, L. P., Maresz, K. & Dittel, B. N. (2005) Microglial cell activation and proliferation precedes the onset of CNS autoimmunity. *J Neurosci Res*, 81, 374-89.
- Prendergast, C. T. & Anderton, S. M. (2009) Immune cell entry to central nervous system--current understanding and prospective therapeutic targets. *Endocr Metab Immune Disord Drug Targets*, 9, 315-27.
- Provoost, S., Maes, T., van Durme, Y. M., Gevaert, P., Bachert, C., Schmidt-Weber, C. B., Brusselle, G. G., Joos, G. F. & Tournoy, K. G. (2009) Decreased FOXP3 protein expression in patients with asthma. *Allergy*, 64, 1539-46.
- Putnam, A. L., Brusko, T. M., Lee, M. R., Liu, W., Szot, G. L., Ghosh, T., Atkinson, M. A. & Bluestone, J. A. (2009) Expansion of human regulatory T-cells from patients with type 1 diabetes. *Diabetes*, 58, 652-62.
- Qin, S., Cobbold, S. P., Pope, H., Elliott, J., Kioussis, D., Davies, J. & Waldmann, H. (1993) "Infectious" transplantation tolerance. *Science*, 259, 974-7.
- Qureshi, O. S., Zheng, Y., Nakamura, K., Attridge, K., Manzotti, C., Schmidt, E. M., Baker, J., Jeffery, L. E., Kaur, S., Briggs, Z., Hou, T. Z., Futter, C. E., Anderson, G., Walker, L. S. & Sansom, D. M. (2011) Trans-endocytosis of CD80 and CD86: a molecular basis for the cell-extrinsic function of CTLA-4. *Science*, 332, 600-3.
- Ransohoff, R. M., Kivisakk, P. & Kidd, G. (2003) Three or more routes for leukocyte migration into the central nervous system. *Nat Rev Immunol*, 3, 569-81.
- Read, S., Malmstrom, V. & Powrie, F. (2000) Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation. *J Exp Med*, 192, 295-302.
- Reboldi, A., Coisne, C., Baumjohann, D., Benvenuto, F., Bottinelli, D., Lira, S., Uccelli, A., Lanzavecchia, A., Engelhardt, B. & Sallusto, F. (2009) C-C chemokine receptor 6-regulated entry of TH-17 cells into the CNS through the choroid plexus is required for the initiation of EAE. *Nat Immunol*, 10, 514-23.
- Reiser, J. B., Darnault, C., Gregoire, C., Mosser, T., Mazza, G., Kearney, A., van der Merwe, P. A., Fontecilla-Camps, J. C., Housset, D. & Malissen, B. (2003) CDR3 loop flexibility contributes to the degeneracy of TCR recognition. *Nat Immunol*, 4, 241-7.
- Ren, X., Ye, F., Jiang, Z., Chu, Y., Xiong, S. & Wang, Y. (2007) Involvement of cellular death in TRAIL/DR5-dependent suppression induced by CD4(+)CD25(+) regulatory T cells. *Cell Death Differ*, 14, 2076-84.

- Renno, T., Krakowski, M., Piccirillo, C., Lin, J. Y. & Owens, T. (1995) TNF- α expression by resident microglia and infiltrating leukocytes in the central nervous system of mice with experimental allergic encephalomyelitis. Regulation by Th1 cytokines. *J Immunol*, 154, 944-53.
- Rice, G. P., Hartung, H. P. & Calabresi, P. A. (2005) Anti- α 4 integrin therapy for multiple sclerosis: mechanisms and rationale. *Neurology*, 64, 1336-42.
- Robinson, D., Hamid, Q., Bentley, A., Ying, S., Kay, A. B. & Durham, S. R. (1993) Activation of CD4⁺ T cells, increased TH2-type cytokine mRNA expression, and eosinophil recruitment in bronchoalveolar lavage after allergen inhalation challenge in patients with atopic asthma. *J Allergy Clin Immunol*, 92, 313-24.
- Robinson, D. S., Hamid, Q., Ying, S., Tsicopoulos, A., Barkans, J., Bentley, A. M., Corrigan, C., Durham, S. R. & Kay, A. B. (1992) Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. *N Engl J Med*, 326, 298-304.
- Rocha, B., Tanchot, C. & Von Boehmer, H. (1993) Clonal anergy blocks in vivo growth of mature T cells and can be reversed in the absence of antigen. *J Exp Med*, 177, 1517-21.
- Rodriguez, E., Baurecht, H., Herberich, E., Wagenpfeil, S., Brown, S. J., Cordell, H. J., Irvine, A. D. & Weidinger, S. (2009) Meta-analysis of filaggrin polymorphisms in eczema and asthma: robust risk factors in atopic disease. *J Allergy Clin Immunol*, 123, 1361-70 e7.
- Roncarolo, M. G., Gregori, S., Battaglia, M., Bacchetta, R., Fleischhauer, K. & Levings, M. K. (2006) Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. *Immunol Rev*, 212, 28-50.
- Ruuls, S. R., Bauer, J., Sontrop, K., Huitinga, I., t Hart, B. A. & Dijkstra, C. D. (1995) Reactive oxygen species are involved in the pathogenesis of experimental allergic encephalomyelitis in Lewis rats. *J Neuroimmunol*, 56, 207-17.
- Ryan, K. R., Patel, S. D., Stephens, L. A. & Anderton, S. M. (2007) Death, adaptation and regulation: the three pillars of immune tolerance restrict the risk of autoimmune disease caused by molecular mimicry. *J Autoimmun*, 29, 262-71.
- Sahu, N., Morales, J. L., Fowell, D. & August, A. (2010) Modeling susceptibility versus resistance in allergic airway disease reveals regulation by Tec kinase Itk. *PLoS One*, 5, e11348.
- Sakaguchi, S., Fukuma, K., Kuribayashi, K. & Masuda, T. (1985) Organ-specific autoimmune diseases induced in mice by elimination of T cell subset. I. Evidence for the active participation of T cells in natural self-tolerance; deficit of a T cell subset as a possible cause of autoimmune disease. *J Exp Med*, 161, 72-87.
- Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M. & Toda, M. (1995) Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor α -chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol*, 155, 1151-64.
- Sakaguchi, S., Yamaguchi, T., Nomura, T. & Ono, M. (2008) Regulatory T cells and immune tolerance. *Cell*, 133, 775-87.

- Salgame, P. (2005) Host innate and Th1 responses and the bacterial factors that control *Mycobacterium tuberculosis* infection. *Curr Opin Immunol*, 17, 374-80.
- Schaerli, P., Willmann, K., Lang, A. B., Lipp, M., Loetscher, P. & Moser, B. (2000) CXC chemokine receptor 5 expression defines follicular homing T cells with B cell helper function. *J Exp Med*, 192, 1553-62.
- Schmidt, A., Oberle, N. & Krammer, P. H. (2012) Molecular mechanisms of treg-mediated T cell suppression. *Front Immunol*, 3, 51.
- Schulz, O., Sewell, H. F. & Shakib, F. (1998) Proteolytic cleavage of CD25, the alpha subunit of the human T cell interleukin 2 receptor, by Der p 1, a major mite allergen with cysteine protease activity. *J Exp Med*, 187, 271-5.
- Schwartz, R. H. (2003) T cell anergy. *Annu Rev Immunol*, 21, 305-34.
- Scott, B., Bluthmann, H., Teh, H. S. & von Boehmer, H. (1989) The generation of mature T cells requires interaction of the alpha beta T-cell receptor with major histocompatibility antigens. *Nature*, 338, 591-3.
- Seddiki, N., Santner-Nanan, B., Martinson, J., Zaunders, J., Sasson, S., Landay, A., Solomon, M., Selby, W., Alexander, S. I., Nanan, R., Kelleher, A. & Fazekas de St Groth, B. (2006) Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. *J Exp Med*, 203, 1693-700.
- Seddon, B. & Mason, D. (2000) The third function of the thymus. *Immunol Today*, 21, 95-9.
- Seder, R. A., Gazzinelli, R., Sher, A. & Paul, W. E. (1993) Interleukin 12 acts directly on CD4+ T cells to enhance priming for interferon gamma production and diminishes interleukin 4 inhibition of such priming. *Proc Natl Acad Sci U S A*, 90, 10188-92.
- Sellner, J., Kraus, J., Awad, A., Milo, R., Hemmer, B. & Stuve, O. (2011) The increasing incidence and prevalence of female multiple sclerosis--a critical analysis of potential environmental factors. *Autoimmun Rev*, 10, 495-502.
- Selvaraj, R. K. & Geiger, T. L. (2008) Mitigation of experimental allergic encephalomyelitis by TGF-beta induced Foxp3+ regulatory T lymphocytes through the induction of anergy and infectious tolerance. *J Immunol*, 180, 2830-8.
- Sharpe, A. H. & Freeman, G. J. (2002) The B7-CD28 superfamily. *Nat Rev Immunol*, 2, 116-26.
- Shevach, E. M. (2002) CD4+ CD25+ suppressor T cells: more questions than answers. *Nat Rev Immunol*, 2, 389-400.
- Shevach, E. M. (2009) Mechanisms of foxp3+ T regulatory cell-mediated suppression. *Immunity*, 30, 636-45.
- Shi, G., Cox, C. A., Vistica, B. P., Tan, C., Wawrousek, E. F. & Gery, I. (2008) Phenotype switching by inflammation-inducing polarized Th17 cells, but not by Th1 cells. *J Immunol*, 181, 7205-13.
- Shi, Y., Evans, J. E. & Rock, K. L. (2003) Molecular identification of a danger signal that alerts the immune system to dying cells. *Nature*, 425, 516-21.
- Shi, Y. & Massague, J. (2003) Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell*, 113, 685-700.

- Shiina, T., Inoko, H. & Kulski, J. K. (2004) An update of the HLA genomic region, locus information and disease associations: 2004. *Tissue Antigens*, 64, 631-49.
- Shimizu, J., Yamazaki, S., Takahashi, T., Ishida, Y. & Sakaguchi, S. (2002) Stimulation of CD25(+)CD4(+) regulatory T cells through GITR breaks immunological self-tolerance. *Nat Immunol*, 3, 135-42.
- Siewert, C., Menning, A., Dudda, J., Siegmund, K., Lauer, U., Floess, S., Campbell, D. J., Hamann, A. & Huehn, J. (2007) Induction of organ-selective CD4+ regulatory T cell homing. *Eur J Immunol*, 37, 978-89.
- Silva, M. T. (2010) When two is better than one: macrophages and neutrophils work in concert in innate immunity as complementary and cooperative partners of a myeloid phagocyte system. *J Leukoc Biol*, 87, 93-106.
- Singh, N. J. & Schwartz, R. H. (2003) The strength of persistent antigenic stimulation modulates adaptive tolerance in peripheral CD4+ T cells. *J Exp Med*, 198, 1107-17.
- Snapper, C. M. & Paul, W. E. (1987) Interferon-gamma and B cell stimulatory factor-1 reciprocally regulate Ig isotype production. *Science*, 236, 944-7.
- Soilu-Hanninen, M., Airas, L., Mononen, I., Heikkila, A., Viljanen, M. & Hanninen, A. (2005) 25-Hydroxyvitamin D levels in serum at the onset of multiple sclerosis. *Mult Scler*, 11, 266-71.
- Sokol, C. L., Chu, N. Q., Yu, S., Nish, S. A., Laufer, T. M. & Medzhitov, R. (2009) Basophils function as antigen-presenting cells for an allergen-induced T helper type 2 response. *Nat Immunol*, 10, 713-20.
- Soldan, S. S., Berti, R., Salem, N., Secchiero, P., Flamand, L., Calabresi, P. A., Brennan, M. B., Maloni, H. W., McFarland, H. F., Lin, H. C., Patnaik, M. & Jacobson, S. (1997) Association of human herpes virus 6 (HHV-6) with multiple sclerosis: increased IgM response to HHV-6 early antigen and detection of serum HHV-6 DNA. *Nat Med*, 3, 1394-7.
- Sorensen, T. L., Tani, M., Jensen, J., Pierce, V., Lucchinetti, C., Folcik, V. A., Qin, S., Rottman, J., Sellebjerg, F., Strieter, R. M., Frederiksen, J. L. & Ransohoff, R. M. (1999) Expression of specific chemokines and chemokine receptors in the central nervous system of multiple sclerosis patients. *J Clin Invest*, 103, 807-15.
- Sospedra, M. & Martin, R. (2005) Immunology of multiple sclerosis. *Annu Rev Immunol*, 23, 683-747.
- Spach, K. M. & Hayes, C. E. (2005) Vitamin D3 confers protection from autoimmune encephalomyelitis only in female mice. *J Immunol*, 175, 4119-26.
- Spach, K. M., Nashold, F. E., Dittel, B. N. & Hayes, C. E. (2006) IL-10 signaling is essential for 1,25-dihydroxyvitamin D3-mediated inhibition of experimental autoimmune encephalomyelitis. *J Immunol*, 177, 6030-7.
- Sperling, A. I., Auger, J. A., Ehst, B. D., Rulifson, I. C., Thompson, C. B. & Bluestone, J. A. (1996) CD28/B7 interactions deliver a unique signal to naive T cells that regulates cell survival but not early proliferation. *J Immunol*, 157, 3909-17.
- Sporici, R. & Issekutz, T. B. (2010) CXCR3 blockade inhibits T-cell migration into the CNS during EAE and prevents development of adoptively transferred, but not actively induced, disease. *Eur J Immunol*, 40, 2751-61.

- Steinman, R. M. (1991) The dendritic cell system and its role in immunogenicity. *Annu Rev Immunol*, 9, 271-96.
- Stephens, G. L., Andersson, J. & Shevach, E. M. (2007) Distinct subsets of FoxP3+ regulatory T cells participate in the control of immune responses. *J Immunol*, 178, 6901-11.
- Stephens, L. A., Malpass, K. H. & Anderton, S. M. (2009) Curing CNS autoimmune disease with myelin-reactive Foxp3+ Treg. *Eur J Immunol*, 39, 1108-17.
- Stock, P., Akbari, O., Berry, G., Freeman, G. J., Dekruyff, R. H. & Umetsu, D. T. (2004) Induction of T helper type 1-like regulatory cells that express Foxp3 and protect against airway hyper-reactivity. *Nat Immunol*, 5, 1149-56.
- Stoll, S., Delon, J., Brotz, T. M. & Germain, R. N. (2002) Dynamic imaging of T cell-dendritic cell interactions in lymph nodes. *Science*, 296, 1873-6.
- Stone, K. D., Prussin, C. & Metcalfe, D. D. (2010) IgE, mast cells, basophils, and eosinophils. *J Allergy Clin Immunol*, 125, S73-80.
- Strachan, D. P. (1989) Hay fever, hygiene, and household size. *Bmj*, 299, 1259-60.
- Strauss, L., Whiteside, T. L., Knights, A., Bergmann, C., Knuth, A. & Zippelius, A. (2007) Selective survival of naturally occurring human CD4+CD25+Foxp3+ regulatory T cells cultured with rapamycin. *J Immunol*, 178, 320-9.
- Stritesky, G. L., Yeh, N. & Kaplan, M. H. (2008) IL-23 promotes maintenance but not commitment to the Th17 lineage. *J Immunol*, 181, 5948-55.
- Stromnes, I. M. & Goverman, J. M. (2006) Passive induction of experimental allergic encephalomyelitis. *Nat Protoc*, 1, 1952-60.
- Suffner, J., Hochweller, K., Kuhnle, M. C., Li, X., Kroczeck, R. A., Garbi, N. & Hammerling, G. J. (2010) Dendritic cells support homeostatic expansion of Foxp3+ regulatory T cells in Foxp3.LuciDTR mice. *J Immunol*, 184, 1810-20.
- Sugimoto, T., Ishikawa, Y., Yoshimoto, T., Hayashi, N., Fujimoto, J. & Nakanishi, K. (2004) Interleukin 18 acts on memory T helper cells type 1 to induce airway inflammation and hyperresponsiveness in a naive host mouse. *J Exp Med*, 199, 535-45.
- Sun, D., Whitaker, J. N., Huang, Z., Liu, D., Coleclough, C., Wekerle, H. & Raine, C. S. (2001) Myelin antigen-specific CD8+ T cells are encephalitogenic and produce severe disease in C57BL/6 mice. *J Immunol*, 166, 7579-87.
- Sun, J. B., Flach, C. F., Czerkinsky, C. & Holmgren, J. (2008) B lymphocytes promote expansion of regulatory T cells in oral tolerance: powerful induction by antigen coupled to cholera toxin B subunit. *J Immunol*, 181, 8278-87.
- Szabo, S. J., Kim, S. T., Costa, G. L., Zhang, X., Fathman, C. G. & Glimcher, L. H. (2000) A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell*, 100, 655-69.
- Tadokoro, C. E., Shakhar, G., Shen, S., Ding, Y., Lino, A. C., Maraver, A., Lafaille, J. J. & Dustin, M. L. (2006) Regulatory T cells inhibit stable contacts between CD4+ T cells and dendritic cells in vivo. *J Exp Med*, 203, 505-11.
- Tai, X., Cowan, M., Feigenbaum, L. & Singer, A. (2005) CD28 costimulation of developing thymocytes induces Foxp3 expression and regulatory T cell differentiation independently of interleukin 2. *Nat Immunol*, 6, 152-62.
- Takahashi, T., Kuniyasu, Y., Toda, M., Sakaguchi, N., Itoh, M., Iwata, M., Shimizu, J. & Sakaguchi, S. (1998) Immunologic self-tolerance maintained by CD25+CD4+ naturally anergic and suppressive T cells: induction of

- autoimmune disease by breaking their anergic/suppressive state. *Int Immunol*, 10, 1969-80.
- Takahashi, T., Tagami, T., Yamazaki, S., Uede, T., Shimizu, J., Sakaguchi, N., Mak, T. W. & Sakaguchi, S. (2000) Immunologic self-tolerance maintained by CD25(+)CD4(+) regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. *J Exp Med*, 192, 303-10.
- Takeuchi, O. & Akira, S. (2010) Pattern recognition receptors and inflammation. *Cell*, 140, 805-20.
- Tang, Q., Henriksen, K. J., Bi, M., Finger, E. B., Szot, G., Ye, J., Masteller, E. L., McDevitt, H., Bonyhadi, M. & Bluestone, J. A. (2004) In vitro-expanded antigen-specific regulatory T cells suppress autoimmune diabetes. *J Exp Med*, 199, 1455-65.
- Teitelbaum, D., Meshorer, A., Hirshfeld, T., Arnon, R. & Sela, M. (1971) Suppression of experimental allergic encephalomyelitis by a synthetic polypeptide. *Eur J Immunol*, 1, 242-8.
- Thangada, S., Khanna, K. M., Blaho, V. A., Oo, M. L., Im, D. S., Guo, C., Lefrancois, L. & Hla, T. (2010) Cell-surface residence of sphingosine 1-phosphate receptor 1 on lymphocytes determines lymphocyte egress kinetics. *J Exp Med*, 207, 1475-83.
- Thompson, C. B., Lindsten, T., Ledbetter, J. A., Kunkel, S. L., Young, H. A., Emerson, S. G., Leiden, J. M. & June, C. H. (1989) CD28 activation pathway regulates the production of multiple T-cell-derived lymphokines/cytokines. *Proc Natl Acad Sci U S A*, 86, 1333-7.
- Thornton, A. M., Korty, P. E., Tran, D. Q., Wohlfert, E. A., Murray, P. E., Belkaid, Y. & Shevach, E. M. (2010) Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3⁺ T regulatory cells. *J Immunol*, 184, 3433-41.
- Thornton, A. M. & Shevach, E. M. (1998) CD4⁺CD25⁺ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J Exp Med*, 188, 287-96.
- Thornton, A. M. & Shevach, E. M. (2000) Suppressor effector function of CD4⁺CD25⁺ immunoregulatory T cells is antigen nonspecific. *J Immunol*, 164, 183-90.
- Thorstenson, K. M. & Khoruts, A. (2001) Generation of anergic and potentially immunoregulatory CD25⁺CD4⁺ T cells in vivo after induction of peripheral tolerance with intravenous or oral antigen. *J Immunol*, 167, 188-95.
- Ting, C. N., Olson, M. C., Barton, K. P. & Leiden, J. M. (1996) Transcription factor GATA-3 is required for development of the T-cell lineage. *Nature*, 384, 474-8.
- Traidl-Hoffmann, C., Jakob, T. & Behrendt, H. (2009) Determinants of allergenicity. *J Allergy Clin Immunol*, 123, 558-66.
- Tran, E. H., Hardin-Pouzet, H., Verge, G. & Owens, T. (1997) Astrocytes and microglia express inducible nitric oxide synthase in mice with experimental allergic encephalomyelitis. *J Neuroimmunol*, 74, 121-9.
- Tran, E. H., Hoekstra, K., van Rooijen, N., Dijkstra, C. D. & Owens, T. (1998) Immune invasion of the central nervous system parenchyma and experimental allergic encephalomyelitis, but not leukocyte extravasation from blood, are prevented in macrophage-depleted mice. *J Immunol*, 161, 3767-75.

- Trifari, S., Kaplan, C. D., Tran, E. H., Crellin, N. K. & Spits, H. (2009) Identification of a human helper T cell population that has abundant production of interleukin 22 and is distinct from T(H)-17, T(H)1 and T(H)2 cells. *Nat Immunol*, 10, 864-71.
- Trompette, A., Divanovic, S., Visintin, A., Blanchard, C., Hegde, R. S., Madan, R., Thorne, P. S., Wills-Karp, M., Gioannini, T. L., Weiss, J. P. & Karp, C. L. (2009) Allergenicity resulting from functional mimicry of a Toll-like receptor complex protein. *Nature*, 457, 585-8.
- Tu, W., Lau, Y. L., Zheng, J., Liu, Y., Chan, P. L., Mao, H., Dionis, K., Schneider, P. & Lewis, D. B. (2008) Efficient generation of human alloantigen-specific CD4⁺ regulatory T cells from naive precursors by CD40-activated B cells. *Blood*, 112, 2554-62.
- Tzartos, J. S., Friese, M. A., Craner, M. J., Palace, J., Newcombe, J., Esiri, M. M. & Fugger, L. (2008) Interleukin-17 production in central nervous system-infiltrating T cells and glial cells is associated with active disease in multiple sclerosis. *Am J Pathol*, 172, 146-55.
- Valencia, X., Stephens, G., Goldbach-Mansky, R., Wilson, M., Shevach, E. M. & Lipsky, P. E. (2006) TNF downmodulates the function of human CD4⁺CD25^{hi} T-regulatory cells. *Blood*, 108, 253-61.
- van der Velden, V. H. (1998) Glucocorticoids: mechanisms of action and anti-inflammatory potential in asthma. *Mediators Inflamm*, 7, 229-37.
- van Santen, H. M., Benoist, C. & Mathis, D. (2004) Number of T reg cells that differentiate does not increase upon encounter of agonist ligand on thymic epithelial cells. *J Exp Med*, 200, 1221-30.
- Vanderlugt, C. L. & Miller, S. D. (2002) Epitope spreading in immune-mediated diseases: implications for immunotherapy. *Nat Rev Immunol*, 2, 85-95.
- Veldhoen, M., Moncrieffe, H., Hocking, R. J., Atkins, C. J. & Stockinger, B. (2006) Modulation of dendritic cell function by naive and regulatory CD4⁺ T cells. *J Immunol*, 176, 6202-10.
- Veldhoen, M., Uyttenhove, C., van Snick, J., Helmby, H., Westendorf, A., Buer, J., Martin, B., Wilhelm, C. & Stockinger, B. (2008) Transforming growth factor-beta 'reprograms' the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset. *Nat Immunol*, 9, 1341-6.
- Vella, A. T., McCormack, J. E., Linsley, P. S., Kappler, J. W. & Marrack, P. (1995) Lipopolysaccharide interferes with the induction of peripheral T cell death. *Immunity*, 2, 261-70.
- Venken, K., Hellings, N., Hensen, K., Rummens, J. L., Medaer, R., D'Hooghe M, B., Dubois, B., Raus, J. & Stinissen, P. (2006) Secondary progressive in contrast to relapsing-remitting multiple sclerosis patients show a normal CD4⁺CD25⁺ regulatory T-cell function and FOXP3 expression. *J Neurosci Res*, 83, 1432-46.
- Vieira, P. L., Christensen, J. R., Minaee, S., O'Neill, E. J., Barrat, F. J., Boonstra, A., Barthlott, T., Stockinger, B., Wraith, D. C. & O'Garra, A. (2004) IL-10-secreting regulatory T cells do not express Foxp3 but have comparable regulatory function to naturally occurring CD4⁺CD25⁺ regulatory T cells. *J Immunol*, 172, 5986-93.

- Viglietta, V., Baecher-Allan, C., Weiner, H. L. & Hafler, D. A. (2004) Loss of functional suppression by CD4⁺CD25⁺ regulatory T cells in patients with multiple sclerosis. *J Exp Med*, 199, 971-9.
- Villares, R., Cadenas, V., Lozano, M., Almonacid, L., Zaballos, A., Martinez, A. C. & Varona, R. (2009) CCR6 regulates EAE pathogenesis by controlling regulatory CD4⁺ T-cell recruitment to target tissues. *Eur J Immunol*, 39, 1671-81.
- Viola, A. & Lanzavecchia, A. (1996) T cell activation determined by T cell receptor number and tunable thresholds. *Science*, 273, 104-6.
- Vyas, J. M., Van der Veen, A. G. & Ploegh, H. L. (2008) The known unknowns of antigen processing and presentation. *Nat Rev Immunol*, 8, 607-18.
- Waldner, H., Whitters, M. J., Sobel, R. A., Collins, M. & Kuchroo, V. K. (2000) Fulminant spontaneous autoimmunity of the central nervous system in mice transgenic for the myelin proteolipid protein-specific T cell receptor. *Proc Natl Acad Sci U S A*, 97, 3412-7.
- Waldor, M. K., Sriram, S., Hardy, R., Herzenberg, L. A., Herzenberg, L. A., Lanier, L., Lim, M. & Steinman, L. (1985) Reversal of experimental allergic encephalomyelitis with monoclonal antibody to a T-cell subset marker. *Science*, 227, 415-7.
- Walker, C., Bode, E., Boer, L., Hansel, T. T., Blaser, K. & Virchow, J. C., Jr. (1992) Allergic and nonallergic asthmatics have distinct patterns of T-cell activation and cytokine production in peripheral blood and bronchoalveolar lavage. *Am Rev Respir Dis*, 146, 109-15.
- Walker, M. R., Kasprovicz, D. J., Gersuk, V. H., Benard, A., Van Landeghen, M., Buckner, J. H. & Ziegler, S. F. (2003) Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4⁺CD25⁻ T cells. *J Clin Invest*, 112, 1437-43.
- Wan, H., Winton, H. L., Soeller, C., Tovey, E. R., Gruenert, D. C., Thompson, P. J., Stewart, G. A., Taylor, G. W., Garrod, D. R., Cannell, M. B. & Robinson, C. (1999) Der p 1 facilitates transepithelial allergen delivery by disruption of tight junctions. *J Clin Invest*, 104, 123-33.
- Wan, Y. Y. (2010) Multi-tasking of helper T cells. *Immunology*, 130, 166-71.
- Wang, X., Lu, L. & Jiang, S. (2011) Regulatory T cells: customizing for the clinic. *Sci Transl Med*, 3, 83ps19.
- Wang, Y., Souabni, A., Flavell, R. A. & Wan, Y. Y. (2010) An intrinsic mechanism predisposes Foxp3-expressing regulatory T cells to Th2 conversion in vivo. *J Immunol*, 185, 5983-92.
- Wang, Z. E., Reiner, S. L., Zheng, S., Dalton, D. K. & Locksley, R. M. (1994) CD4⁺ effector cells default to the Th2 pathway in interferon gamma-deficient mice infected with *Leishmania major*. *J Exp Med*, 179, 1367-71.
- Warner, J. O. (2004) The early life origins of asthma and related allergic disorders. *Arch Dis Child*, 89, 97-102.
- Weaver, C. T., Hatton, R. D., Mangan, P. R. & Harrington, L. E. (2007) IL-17 family cytokines and the expanding diversity of effector T cell lineages. *Annu Rev Immunol*, 25, 821-52.
- Weber, M. S., Starck, M., Wagenpfeil, S., Meinl, E., Hohlfeld, R. & Farina, C. (2004) Multiple sclerosis: glatiramer acetate inhibits monocyte reactivity in vitro and in vivo. *Brain*, 127, 1370-8.

- Wegmann, M. (2008) Animal models of chronic experimental asthma - strategies for the identification of new therapeutic targets. *J Occup Med Toxicol*, 3 Suppl 1, S4.
- Wei, G., Wei, L., Zhu, J., Zang, C., Hu-Li, J., Yao, Z., Cui, K., Kanno, Y., Roh, T. Y., Watford, W. T., Schones, D. E., Peng, W., Sun, H. W., Paul, W. E., O'Shea, J. J. & Zhao, K. (2009) Global mapping of H3K4me3 and H3K27me3 reveals specificity and plasticity in lineage fate determination of differentiating CD4⁺ T cells. *Immunity*, 30, 155-67.
- Wei, J., Duramad, O., Perng, O. A., Reiner, S. L., Liu, Y. J. & Qin, F. X. (2007) Antagonistic nature of T helper 1/2 developmental programs in opposing peripheral induction of Foxp3⁺ regulatory T cells. *Proc Natl Acad Sci U S A*, 104, 18169-74.
- Weiner, H. L. (2001) Oral tolerance: immune mechanisms and the generation of Th3-type TGF-beta-secreting regulatory cells. *Microbes Infect*, 3, 947-54.
- Welle, M. (1997) Development, significance, and heterogeneity of mast cells with particular regard to the mast cell-specific proteases chymase and tryptase. *J Leukoc Biol*, 61, 233-45.
- Weller, P. F. (1991) The immunobiology of eosinophils. *N Engl J Med*, 324, 1110-8.
- Wells, A. D., Li, X. C., Li, Y., Walsh, M. C., Zheng, X. X., Wu, Z., Nunez, G., Tang, A., Sayegh, M., Hancock, W. W., Strom, T. B. & Turka, L. A. (1999) Requirement for T-cell apoptosis in the induction of peripheral transplantation tolerance. *Nat Med*, 5, 1303-7.
- Wenner, C. A., Guler, M. L., Macatonia, S. E., O'Garra, A. & Murphy, K. M. (1996) Roles of IFN-gamma and IFN-alpha in IL-12-induced T helper cell-1 development. *J Immunol*, 156, 1442-7.
- Williams, L. M. & Rudensky, A. Y. (2007) Maintenance of the Foxp3-dependent developmental program in mature regulatory T cells requires continued expression of Foxp3. *Nat Immunol*, 8, 277-84.
- Wills-Karp, M. (1999) Immunologic basis of antigen-induced airway hyperresponsiveness. *Annu Rev Immunol*, 17, 255-81.
- Wilson, N. J., Boniface, K., Chan, J. R., McKenzie, B. S., Blumenschein, W. M., Mattson, J. D., Basham, B., Smith, K., Chen, T., Morel, F., Lecron, J. C., Kastelein, R. A., Cua, D. J., McClanahan, T. K., Bowman, E. P. & de Waal Malefyt, R. (2007) Development, cytokine profile and function of human interleukin 17-producing helper T cells. *Nat Immunol*, 8, 950-7.
- Wing, K., Onishi, Y., Prieto-Martin, P., Yamaguchi, T., Miyara, M., Fehervari, Z., Nomura, T. & Sakaguchi, S. (2008) CTLA-4 control over Foxp3⁺ regulatory T cell function. *Science*, 322, 271-5.
- Wing, K. & Sakaguchi, S. (2010) Regulatory T cells exert checks and balances on self tolerance and autoimmunity. *Nat Immunol*, 11, 7-13.
- Wohlfert, E. A., Grainger, J. R., Bouladoux, N., Konkel, J. E., Oldenhove, G., Ribeiro, C. H., Hall, J. A., Yagi, R., Naik, S., Bhairavabhotla, R., Paul, W. E., Bosselut, R., Wei, G., Zhao, K., Oukka, M., Zhu, J. & Belkaid, Y. (2011) GATA3 controls Foxp3(+) regulatory T cell fate during inflammation in mice. *J Clin Invest*, 121, 4503-15.
- Wolf, S. D., Dittel, B. N., Hardardottir, F. & Janeway, C. A., Jr. (1996) Experimental autoimmune encephalomyelitis induction in genetically B cell-deficient mice. *J Exp Med*, 184, 2271-8.

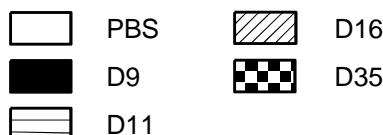
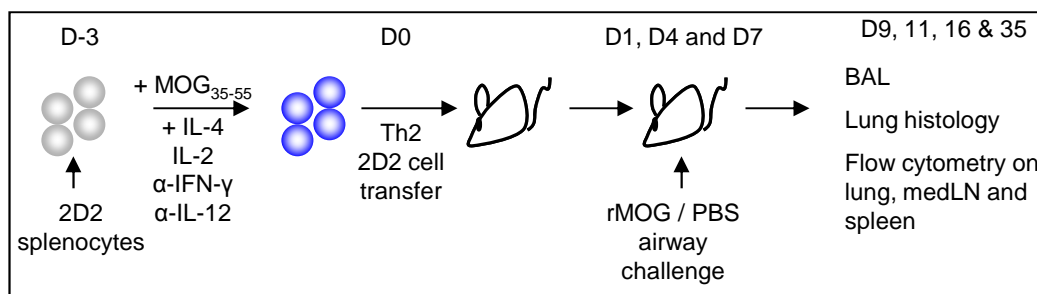
- Wong, J., Obst, R., Correia-Neves, M., Losyev, G., Mathis, D. & Benoist, C. (2007) Adaptation of TCR repertoires to self-peptides in regulatory and nonregulatory CD4+ T cells. *J Immunol*, 178, 7032-41.
- Woolcock, A., Lundback, B., Ringdal, N. & Jacques, L. A. (1996) Comparison of addition of salmeterol to inhaled steroids with doubling of the dose of inhaled steroids. *Am J Respir Crit Care Med*, 153, 1481-8.
- Wu, Y., Borde, M., Heissmeyer, V., Feuerer, M., Lapan, A. D., Stroud, J. C., Bates, D. L., Guo, L., Han, A., Ziegler, S. F., Mathis, D., Benoist, C., Chen, L. & Rao, A. (2006) FOXP3 controls regulatory T cell function through cooperation with NFAT. *Cell*, 126, 375-87.
- Wurtz, O., Bajenoff, M. & Guerder, S. (2004) IL-4-mediated inhibition of IFN-gamma production by CD4+ T cells proceeds by several developmentally regulated mechanisms. *Int Immunol*, 16, 501-8.
- Xu, L., Kitani, A., Fuss, I. & Strober, W. (2007) Cutting edge: regulatory T cells induce CD4+CD25-Foxp3- T cells or are self-induced to become Th17 cells in the absence of exogenous TGF-beta. *J Immunol*, 178, 6725-9.
- Xu, W., Lan, Q., Chen, M., Chen, H., Zhu, N., Zhou, X., Wang, J., Fan, H., Yan, C. S., Kuang, J. L., Warburton, D., Togbe, D., Ryffel, B., Zheng, S. G. & Shi, W. (2012) Adoptive transfer of induced-treg cells effectively attenuates murine airway allergic inflammation. *PLoS One*, 7, e40314.
- Xystrakis, E., Dejean, A. S., Bernard, I., Druet, P., Liblau, R., Gonzalez-Dunia, D. & Saoudi, A. (2004) Identification of a novel natural regulatory CD8 T-cell subset and analysis of its mechanism of regulation. *Blood*, 104, 3294-301.
- Yang, I. A., Fong, K. M., Holgate, S. T. & Holloway, J. W. (2006) The role of Toll-like receptors and related receptors of the innate immune system in asthma. *Curr Opin Allergy Clin Immunol*, 6, 23-8.
- Yang, M., Hogan, S. P., Henry, P. J., Matthaei, K. I., McKenzie, A. N., Young, I. G., Rothenberg, M. E. & Foster, P. S. (2001) Interleukin-13 mediates airways hyperreactivity through the IL-4 receptor-alpha chain and STAT-6 independently of IL-5 and eotaxin. *Am J Respir Cell Mol Biol*, 25, 522-30.
- Yang, X. O., Nurieva, R., Martinez, G. J., Kang, H. S., Chung, Y., Pappu, B. P., Shah, B., Chang, S. H., Schluns, K. S., Watowich, S. S., Feng, X. H., Jetten, A. M. & Dong, C. (2008a) Molecular antagonism and plasticity of regulatory and inflammatory T cell programs. *Immunity*, 29, 44-56.
- Yang, X. O., Pappu, B. P., Nurieva, R., Akimzhanov, A., Kang, H. S., Chung, Y., Ma, L., Shah, B., Panopoulos, A. D., Schluns, K. S., Watowich, S. S., Tian, Q., Jetten, A. M. & Dong, C. (2008b) T helper 17 lineage differentiation is programmed by orphan nuclear receptors ROR alpha and ROR gamma. *Immunity*, 28, 29-39.
- Yednock, T. A., Cannon, C., Fritz, L. C., Sanchez-Madrid, F., Steinman, L. & Karin, N. (1992) Prevention of experimental autoimmune encephalomyelitis by antibodies against alpha 4 beta 1 integrin. *Nature*, 356, 63-6.
- Yin, Y., Wang, X. X. & Mariuzza, R. A. (2012) Crystal structure of a complete ternary complex of T-cell receptor, peptide-MHC, and CD4. *Proc Natl Acad Sci U S A*, 109, 5405-10.
- Yoshimoto, T., Takeda, K., Tanaka, T., Ohkusu, K., Kashiwamura, S., Okamura, H., Akira, S. & Nakanishi, K. (1998) IL-12 up-regulates IL-18 receptor

- expression on T cells, Th1 cells, and B cells: synergism with IL-18 for IFN- γ production. *J Immunol*, 161, 3400-7.
- Yu, P., Gregg, R. K., Bell, J. J., Ellis, J. S., Divekar, R., Lee, H. H., Jain, R., Waldner, H., Hardaway, J. C., Collins, M., Kuchroo, V. K. & Zaghoulani, H. (2005) Specific T regulatory cells display broad suppressive functions against experimental allergic encephalomyelitis upon activation with cognate antigen. *J Immunol*, 174, 6772-80.
- Yu, P., Haymaker, C. L., Divekar, R. D., Ellis, J. S., Hardaway, J., Jain, R., Tartar, D. M., Hoeman, C. M., Cascio, J. A., Ostermeier, A. & Zaghoulani, H. (2008) Fetal exposure to high-avidity TCR ligand enhances expansion of peripheral T regulatory cells. *J Immunol*, 181, 73-80.
- Yuan, Q., Bromley, S. K., Means, T. K., Jones, K. J., Hayashi, F., Bhan, A. K. & Luster, A. D. (2007) CCR4-dependent regulatory T cell function in inflammatory bowel disease. *J Exp Med*, 204, 1327-34.
- Zal, T., Volkman, A. & Stockinger, B. (1994) Mechanisms of tolerance induction in major histocompatibility complex class II-restricted T cells specific for a blood-borne self-antigen. *J Exp Med*, 180, 2089-99.
- Zamvil, S., Nelson, P., Trotter, J., Mitchell, D., Knobler, R., Fritz, R. & Steinman, L. (1985) T-cell clones specific for myelin basic protein induce chronic relapsing paralysis and demyelination. *Nature*, 317, 355-8.
- Zamvil, S. S., Mitchell, D. J., Moore, A. C., Kitamura, K., Steinman, L. & Rothbard, J. B. (1986) T-cell epitope of the autoantigen myelin basic protein that induces encephalomyelitis. *Nature*, 324, 258-60.
- Zhang, D. H., Cohn, L., Ray, P., Bottomly, K. & Ray, A. (1997) Transcription factor GATA-3 is differentially expressed in murine Th1 and Th2 cells and controls Th2-specific expression of the interleukin-5 gene. *J Biol Chem*, 272, 21597-603.
- Zhang, D. H., Yang, L., Cohn, L., Parkyn, L., Homer, R., Ray, P. & Ray, A. (1999) Inhibition of allergic inflammation in a murine model of asthma by expression of a dominant-negative mutant of GATA-3. *Immunity*, 11, 473-82.
- Zhang, H., Podojil, J. R., Chang, J., Luo, X. & Miller, S. D. (2010) TGF- β -induced myelin peptide-specific regulatory T cells mediate antigen-specific suppression of induction of experimental autoimmune encephalomyelitis. *J Immunol*, 184, 6629-36.
- Zhang, X., Koldzic, D. N., Izikson, L., Reddy, J., Nazareno, R. F., Sakaguchi, S., Kuchroo, V. K. & Weiner, H. L. (2004) IL-10 is involved in the suppression of experimental autoimmune encephalomyelitis by CD25⁺CD4⁺ regulatory T cells. *Int Immunol*, 16, 249-56.
- Zhang, Z., Hener, P., Frossard, N., Kato, S., Metzger, D., Li, M. & Chambon, P. (2009) Thymic stromal lymphopoietin overproduced by keratinocytes in mouse skin aggravates experimental asthma. *Proc Natl Acad Sci U S A*, 106, 1536-41.
- Zheng, W. & Flavell, R. A. (1997) The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. *Cell*, 89, 587-96.
- Zheng, Y., Chaudhry, A., Kas, A., deRoos, P., Kim, J. M., Chu, T. T., Corcoran, L., Treuting, P., Klein, U. & Rudensky, A. Y. (2009) Regulatory T-cell suppressor program co-opts transcription factor IRF4 to control T(H)2 responses. *Nature*, 458, 351-6.

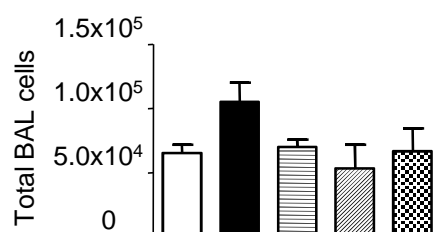
- Zheng, Y., Josefowicz, S. Z., Kas, A., Chu, T. T., Gavin, M. A. & Rudensky, A. Y. (2007) Genome-wide analysis of Foxp3 target genes in developing and mature regulatory T cells. *Nature*, 445, 936-40.
- Zhou, L., Ivanov, II, Spolski, R., Min, R., Shenderov, K., Egawa, T., Levy, D. E., Leonard, W. J. & Littman, D. R. (2007) IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. *Nat Immunol*, 8, 967-74.
- Zhou, X., Bailey-Bucktrout, S., Jeker, L. T. & Bluestone, J. A. (2009a) Plasticity of CD4(+) FoxP3(+) T cells. *Curr Opin Immunol*, 21, 281-5.
- Zhou, X., Bailey-Bucktrout, S. L., Jeker, L. T., Penaranda, C., Martinez-Llordella, M., Ashby, M., Nakayama, M., Rosenthal, W. & Bluestone, J. A. (2009b) Instability of the transcription factor Foxp3 leads to the generation of pathogenic memory T cells in vivo. *Nat Immunol*, 10, 1000-7.
- Zhu, J., Cote-Sierra, J., Guo, L. & Paul, W. E. (2003) Stat5 activation plays a critical role in Th2 differentiation. *Immunity*, 19, 739-48.
- Zoller, M. (1991) Intrathymic presentation by dendritic cells and macrophages: their role in selecting T cells with specificity for internal and external nominal antigen. *Immunology*, 74, 407-13.
- Zonneveld-Huijssoon, E., Roord, S. T., de Jager, W., Klein, M., Albani, S., Anderton, S. M., Kuis, W., van Wijk, F. & Prakken, B. J. (2011) Bystander suppression of experimental arthritis by nasal administration of a heat shock protein peptide. *Ann Rheum Dis*, 70, 2199-206.

8 Appendix

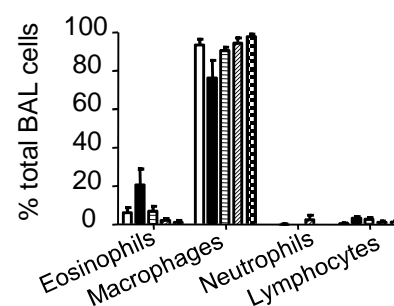
A



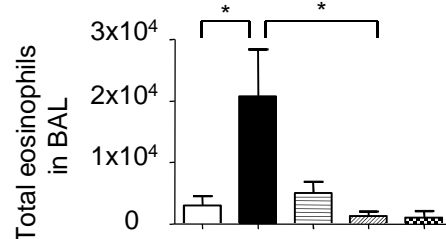
B



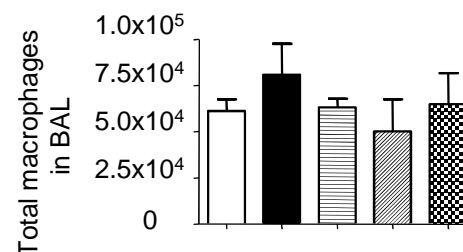
C



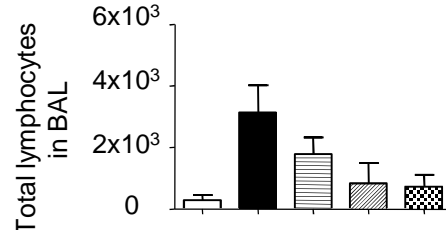
D



E

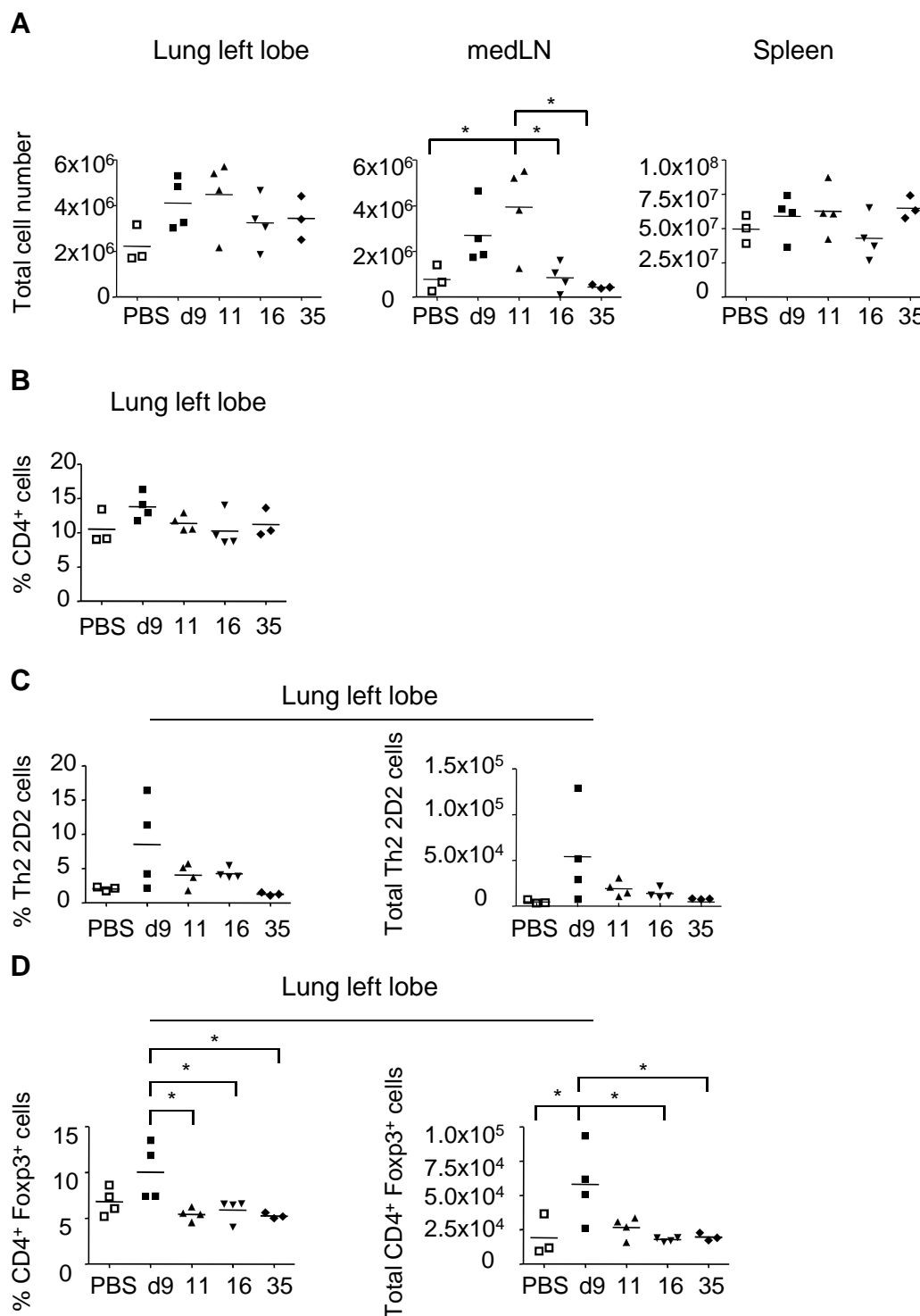


F



Appendix 1 Kinetics of rMOG-induced Th2 2D2-driven mediated AAI.

Traceable Th2-polarised CD4⁺ 2D2 cells were transferred into C57BL/6 mice followed by three intratracheal challenges with rMOG. (A) Experimental scheme. (B) Total cell numbers in BAL. (C) Percentages of cells in BAL. (D) Total numbers of eosinophils, (E) macrophages and (F) lymphocytes in BAL. Data are from one experiment. Data are shown as mean \pm SEM. * $p < 0.05$ as determined by a one-way variance (ANOVA) using Tukey's multiple comparison test.



Appendix 2 The highest percentage and total numbers of Foxp3⁺ CD4⁺ cells is found on day 9.

Experimental approach is shown in Appendix 1. (A) Total cell numbers. (B) Percentages of CD4⁺ cells of all cells in the lung. (C) Percentages of Th2 2D2 cells of all CD4⁺ cells and total cell numbers of Th2 2D2 cells in the lung. (D) Percentages of total CD4⁺ Foxp3⁺ cells of all CD4⁺ cells and total cell numbers of Foxp3⁺ CD4⁺ cells in the lung. Data are from one experiment. *p<0.05 as determined by a one-way variance (ANOVA) using Tukey's multiple comparison test.